AD_____

Award Number: DAMD17-99-1-9028

TITLE: Osteoblast-Prostate Cancer Cell Interaction in Prostate Cancer Bone Metastases

PRINCIPAL INVESTIGATOR: Nora M. Navone, Ph.D.

CONTRACTING ORGANIZATION: University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: August 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | August 2001 | Final (4 Jan 99 - 3 Jul 01) |

**4. TITLE AND SUBTITLE**
Osteoblast-Prostate Cancer Cell Interaction in Prostate Cancer Bone Metastases

**5. FUNDING NUMBERS**
DAMD17-99-1-9028

**6. AUTHOR(S)**
Nora M. Navone, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-Mail: nnavone@mail.mdanderson.org

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
Report contains color

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

**14. SUBJECT TERMS**

**15. NUMBER OF PAGES**
21

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

**Osteoblast-Prostate Cancer Cell Interaction in Prostate Cancer Bone Metastases.** New Investigator Award mechanism for the period 1999-2001

**Final Progress Report**. The progress made during this funding period is summarized below and is included in one reprint.

**Introduction**

Metastases from prostate cancer are characterized by their predilection for bone and their propensity to make more bone. This suggests that prostate cancer cells interact with cells from the osteoblastic lineage. To understand the molecular bases of prostatic bone metastases, we established two prostate cancer cell lines, MDA PCa 2a and MDA PCa 2b (1). These cell lines are derived from a bone metastasis of prostate cancer and have typical features of prostate cancer: they express prostate-specific antigen (PSA) and androgen receptor and their growth is regulated by androgen (1). Using these cell lines, we established and optimized an *in vitro* model of bone metastases from prostate cancer. In this system we co-culture prostate cancer cells with primary mouse osteoblasts (PMOs) in a Boyden chamber-type system so that the cells share medium but are not in physical contact (2). PMOs were isolated from the calvaria of 2- to 4-day-old CD1 mice according to the procedure described in Yang *et al* (2). The PMOs were seeded in multiwell plate, and MDA PCa 2a and MDA PCa 2b cells were seeded in chamber inserts with an 0.4 μm pores (Falcon/Becton Dickinson Labware, Franklin Lakes, NJ). After 24 h of cell culture, the inserts containing MDA PCa 2a and MDA PCa 2b cells were placed into tissue-culture plates containing the PMOs so that the two different cell types shared the culture medium but were not in physical contact. As a growth medium we used α-minimal essential medium (α-MEM) plus 10% fetal bovine serum (FBS) plus 5% BRFF-HPC1 medium (Biological Research Faculty and Facility, Inc., Jamsville, MD) (2)). In all cases PMOs and prostate cancer cells growing alone were used as controls. The medium was changed every 2 days. A similar method was used to co-culture LNCaP, PC3, HeLa and T24 cell lines with PMOs. LNCaP is derived from a lymph node metastasis of prostate cancer, not from a bone metastasis (3). PC3 is derived from a bone metastasis of prostate cancer but do not produce PSA and is not regulated by androgen (3). HeLa and T24, two neoplastic cell lines not of prostate origin, were used as additional controls. Using this system we showed that MDA PCa 2a and MDA PCa 2b cells induce a specific and reproducible increase in osteoblast growth and also induce osteoblast differentiation when the cells share the medium during co-culturing (2).

**Body**

We found evidence of a significant increase in PMO proliferation when these cells were co-cultured with MDA PCa 2a and MDA PCa 2b cells ($p < 0.001$ for both, Fig. 1). In contrast, when PMOs were co-cultured with PC3 cells, the number of PMOs decreased significantly ($p<0.001$). When PMOs were co-cultured with LNCaP cells, no statistically significant proliferative response was found in the PMOs. The number of PMOs also decreased significantly after co-culture with HeLa cells ($p<0.001$), whereas no significant modification was observed with T24 cells (Fig. 1).

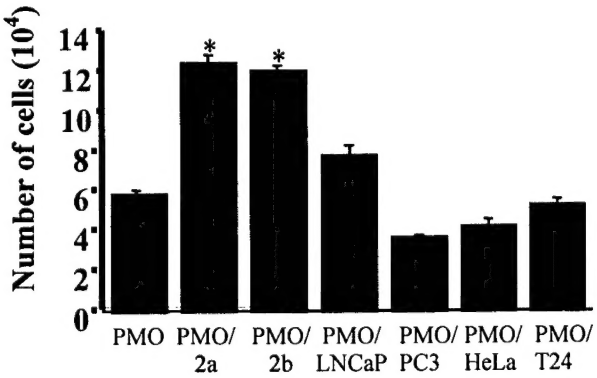



Fig 1. Number of PMOs grown for 4 days either alone (PMO) or in co-culture with MDA PCa 2a (PMO/2a), MDA PCa 2b (PMO/2b), LNCaP (PMO/LNCaP), PC3 (PMO/PC3), HeLa (PMO/HeLa), and T24 (PMO/T24) cells. Each culture or co-culture was assayed six times. Cells were counted in an hematocytometer. Results obtained from three different experiments are expressed as means; bars = SE. * Number of co-cultured PMOs significantly higher than controls ($p< 0.001$). ** Number of co-cultured PMOs significantly lower than controls ($p<0.001$)

**These results suggest that the proliferative response observed with the MDA PCa 2a and MDA PCa 2b bone derived prostate cancer cells is specific.**

Cells isolated from calvaria are osteoblasts at various stages of differentiation. The osteoblast, the bone-forming cell, is a cell of mesenchymal origin that when terminally differentiated produces most of the protein present in the bone extracellular matrix (ECM) and controls the mineralization of the ECM. Regulation of normal bone formation involves sequential expression of growth factors and transcription factors by osteoblasts as they proliferate and ultimately differentiate. *Cbfa1* is the only known osteoblast-specific transcription factor and regulator of osteoblast differentiation (4, 5). To assess whether prostate cancer cells can induce PMOs to differentiate, we assessed expression of *Cbfa1* and its target genes *Osteocalcin* and *Collagen type I* in PMOs grown alone and after 4 days of co-culture with prostate cancer cells. *Osteocalcin* genes are expressed solely in osteoblasts and no other ECM-producing cells (5) and *Collagen Type I* is highly produced by osteoblasts, constitutes more than 90% of the bone ECM proteins and *Cbfa1* is a positive regulator of the osteoblast-specific expression of both *type I collagen* genes (5, 6). As Fig. 2 shows, PMOs grown in the presence of MDA PCa 2b cells for 4 days expressed more *Osteocalcin* and *Cbfa1* than controls did.

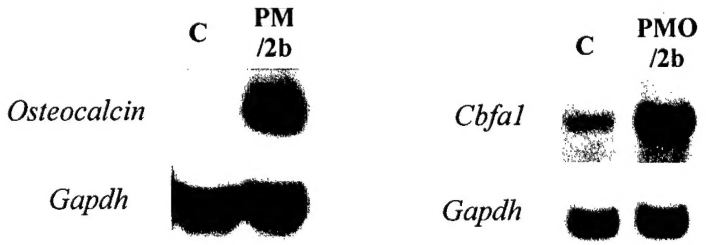



Fig. 2 Northern blot analysis of *Osteocalcin* and *Cbfa1* expression in PMOs grown alone (C), or in co-culture with MDA PCa 2b cells (PMO/2b). Glyceraldhehide 3-phosphate dehydrogenase (*Gapdh)* was used as a loading control

Moreover, PMOs grown in the presence of MDA PCa 2a and MDA PCa 2b cells expressed more mRNA encoding procollagen type I than PMOs growing alone did (2 and Fig 3). Note that other prostate cancer cells did not upregulated the expression of Collagen type I in PMOs.

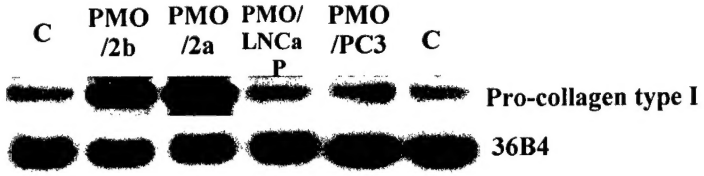



Fig. 3 Northern blot analysis of pro-collagen type I expression in PMOs grown alone, or in co-culture (C) with MDA PCa 2a (PMO/2a), MDA PCa 2b (PMO/2b), PC3 (PMO/PC3), and LNCaP (PMO/LNCaP) prostate cancer cells. *36B4* was used as a loading control

In prolonged culture, primary osteoblasts undergo a defined series of events from proliferation to maturation and express molecular markers of the osteoblast lineage such as osteopontin, alkaline phosphatase, bone sialoprotein, and osteocalcin. Ultimately they are surrounded by mineralized ECM (7-11). We therefore also tested whether prostate cancer cells can influence the ability of PMOs to differentiate when they are placed in prolonged cultures, after the PMOs and prostate cancer cells had been separated. The experiments were performed as follows, after 4 days of co-culture, the inserts containing the prostate cancer cells were removed, and the PMOs were maintained in culture until they reached confluence (day 0). The PMOs were subsequently placed in differentiation medium $\alpha$-MEM plus 10 % FBS, 100 $\mu$g/ml ascorbic acid and 10 nM sodium beta-glycerophosphate) in the presence of BMP-2 (20 ng/ml). Then the PMOs were tested for alkaline phosphatase activity every 2 days for 8 days, and calcified matrix formation was assessed by Von Kossa staining (12) at day 16. Alkaline phosphatase activity and calcified matrix deposition (von Kossa staining) was increase in PMOs grown with MDA PCa 2a, or MDA PCa 2b cells (2). In contrast, alkaline phosphatase activity and von Kossa staining decreased when PMOs were co-cultured with PC3 cells (2). This suggests that PC3 cells secrete molecules that inhibit the osteoblast differentiation program. These results agree with those of others (13, 14) and further validate this system.

**Taken together, these results indicate that the bone derived MDA PCa 2a and MDA PCA2b prostate cancer cells favor molecular events that led to osteoblast differentiation.**

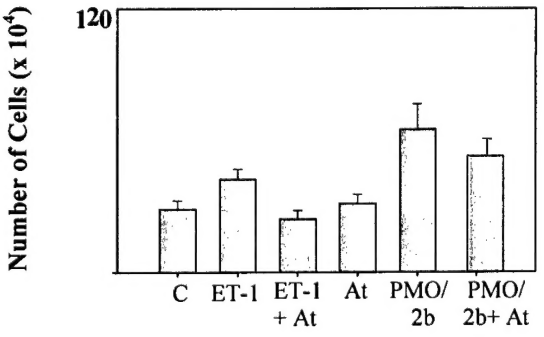
*Cbfa1* is the only known osteoblast-specific transcription factor and regulator of osteoblast differentiation (4, 5, 15). Inactivating mutations in *Cbfa1* in mice and humans prevents osteoblast differentiation and this phenotype is dominant (16). This finding indicates that the function of *Cbfa1* is essential and not redundant with the function of any other gene during bone formation and so that *Cbfa1* is as a master gene in osteoblast differentiation. Although most studies of *Cbfa1* have focused on its role as a transcriptional activator of osteoblast differentiation during embryonic development, **a recent report demonstrated that after birth, *Cbfa1* also controls bone formation after osteoblast differentiation** (17).

Until now, no factor other than *Cbfa1* itself has been shown to interact with the *Cbfa1* promoter and to directly control its activity, although some secreted molecules and transcription factors have been proposed to indirectly regulate *Cbfa1* expression and/or activity (18). Recently, it was proposed that *Cbfa1* transcriptional activity can be modulated at three levels: by increased *Cbfa1* transcripts, by posttranscriptional modifications, and by cofactors (18). **A recent study demonstrated that the mitogen activated protein kinase (MAPKs) pathway induces *Cbfa1* transcriptional activity by phosphorylation** (19).

Studies of prostate cancer cell lines or prostate cancer tissue specimens have identified several factors expressed by prostate cancer cells that can act as direct or indirect osteoblast-stimulating factors, namely PSA (20, 21), urokinase (22), bone morphogenetic proteins (BMP) (23, 24), and endothelin-1 (ET-1) (25).

Multiple studies have identified ET-1 as a molecule involved in the progression of osteoblastic bone metastasis (26-29). The mitogenic effect of ET-1 on osteoblasts is dependent on binding to the $ET_A$ receptor, as the inhibition of the receptor blocks the effects of ET-1 (28). We therefore studied ET-1 production by prostate cancer cells and the effect of an antagonist of its receptor, Atrasentan, on the proliferation and differentiation response of PMOs to MDA PCa 2b cells in our co-culture system. ET-1 was detected in the culture medium of all human prostate cancer cell lines tested. In initial experiments we determined that ET-1 stimulated the growth of PMOs equally over the concentration range tested ($10^{-11}$-$10^{-8}$ M) (data not shown). ET-1 ($10^{-9}$ M) induced PMO growth, whereas Atrasentan ($10^{-5}$ M) reversed the ET-1 induction of PMOs cell growth (Fig. 4). Atrasentan used alone had no significant inhibitory effect on the growth of PMOs (Fig. 4). Furthermore, the stimulating effect of MDA PCa 2b on PMO growth in the co-culture system was partially inhibited by Atrasentan (Fig. 4). In contrast, the stimulation of bone deposition induced by MDA PCa 2b cells was not modified, suggesting that these cells also produce other soluble factors acting in synergy with ET-1 (Fig. 5).

Fig 4. Effect of Atrasentan on MDA PCa 2b-induced growth of PMOs. PMOs were seeded in a 24-well plate, and grown alone (C) or under the following conditions: with ET-1 ($10^{-9}$ M) (ET-1), with ET-1 ($10^{-9}$ M) plus Atrasentan ($10^{-5}$ M) (ET-1+At), with Atrasentan ($10^{-5}$ M) (At). PMOs were also grown in co-culture with MDA PCa 2b cells (PMO/2b) and in co-culture with MDA PCa 2b cells plus Atrasentan ($10^{-5}$ M) (PMO/2b+At). * $p$□0.05 with respect to controls (PMOs grown alone), ** $p$<0.05 with respect to PMOs in co-culture with MDA PCa 2b cells.



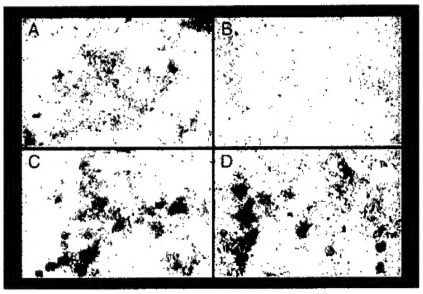



Fig. 5. Atrasentan effect on on MDA PCa 2b-induced calcified matrix deposition. PMOs were cultured with or without MDA PCa 2b cells and with or without Atrasentan ($10^{-5}$ M) in our co-cultured system. The medium was changed after 2 days of co-culture and fresh Atrasentan was added. After 4 days, inserts containing prostate cancer cells were removed, and the PMOs were allowed to grow to confluence. Differentiation medium was then added to the PMOs and changed every 2 days. Recombinant human BMP-2 (20 ng/ml) was used to accelerate e formation of mineralized bone nodules. Bone matrix deposition was assessed by von Kossa's staining at day 12. A, PMO control; B, PMOs + Atrasentan; C, PMOs in co-culture with MDA PCa 2b cells; D, PMOs in co-culture with MDA PCa 2b cells + Atrasentan.

**These results suggest that ET-1-dependent and -independent mechanisms mediate the increase in bone mass in prostate cancer bone metastases**.

Another likely candidate, BMP-2 is not produced by MDA PCa 2b cells assessed by Northern blot analysis of poly(A) RNA and reverse transcription (RT)-polymerase chain reaction (PCR).

Insulin-like growth factors (IGFs) are abundant in human bones and have mitogenic, chemotactic, and antiapoptotic effects on a wide variety of cells, including both prostate cancer cells (30, 31) and osteoblasts (32-34). IGF-I and -II are produced by many cell types, including osteoblasts. In rodents, IGF-II is believed to control intrauterine growth, whereas IGF-I is critical for both prenatal and postnatal growth and is the dominant IGF in adult bone (35). Numerous *in vitro* studies have documented the ability of IGF-I to influence osteoblast growth and differentiation (32-36). In particular, IGF-I induces type I collagen expression in differentiated fetal rat osteoblasts (36). Moreover, targeted overexpression of IGF-I in the osteoblasts of transgenic mice demonstrated an increased in bone formation rate and mineral density (37). Histomorphometric measurements revealed an increase in cancellous bone volume but no change in the total number of osteoblasts, suggesting increased activity of mature osteoblasts (37). Mice carrying null mutations of the genes encoding *Igf1r* are not informative because they die too early (38).

IGF-I and -II exert their action through the Type 1 IGF receptor (IGF1R), a tyrosine kinase receptor structurally related to the insulin receptor. The IGF-II receptor does not transduce a signal, and its primary function appears to be associated with binding and degradation of IGF-II. IGF1R is a dimer that consists of an extracellular ligand binding subunit (the $\alpha$ subunit) and of a transmembrane $\beta$ subunit, which is linked to the $\alpha$ subunit by disulfide bonds. The IGF-1R is a tyrosine kinase and ligand binding causes autophosphorylation of the receptor on tyrosine residues. Autophosphorylation of the receptor is a necessary component for its function. IGF1R is known to be mitogenic in vivo and in vitro, to transmit powerful anti-apoptotic signals, and to induce differentiation in some cell types (39). Signaling pathways for the IGF1R include Shc, which leads to the Ras/Raf/MAPK cascade; the insulin receptor substrate (IRS)-1 and –2; and Crk (35, 39). The immediate substrate Shc, which activates the ras/MAPK pathway, has been shown to favor differentiation in several cell types (39).

Six IGF binding proteins (IGFBPs) regulate the interaction between the IGFs and the IGF1R and are in turn hydrolyzed by a number of proteases (35, 40). IGFBP proteases fall into three major categories: kallikrein-like serine proteases, including PSA, gamma-nerve growth factor, and plasmin; cathepsins, which are intracellular proteinases; and matrix metalloproteinases (MMPs or matrixins) comprise a family of peptide hydrolases that function in tissue remodeling by degrading ECM components such as proteoglycans (35). More than 90% of the circulating IGFs are bound to IGFBP-3. IGFBP-3 modulates the amount of bioavailable free IGFs and inhibits their transfer from the circulation to tissue sites of action (35, 41). Prostate cancer cells has been shown to actively regulate bioavailable IGFs levels by urokinase- and PSA-mediated IGFBP-3 proteolysis (35, 42). Moreover, previous reports have implicated PSA-dependent proteolysis of IGFBP-3 in the osteoblastic phenotype of prostate cancer bone metastases (43-45). A decrease in serum IGFBP-3 in patients with metastatic prostate cancer and a significant negative

correlation between serum PSA and IGFBP-3 were reported (43). In support of those findings, tissue and serum concentrations of PSA vary inversely with IGFBP-3 levels in patients with metastatic prostate cancer (44, 45). Moreover, the IGF signaling pathway seems to play a major role in bone metastasis from prostate cancer, as that pathway may also mediate the mitogenic effect of urokinase-type plasminogen activator on osteoblasts (42).

We then studied expression of PSA and IGFBP-3 in MDA PCa 2a and MDA PCa 2b cells grown alone and after 4 days of co-culture with PMOs. PSA concentration in the growth medium was used as a measure of PSA production by prostate cancer cells and was identical in MDA PCa 2a and MDA PCa 2b cells grown alone and those cultured with PMOs. In contrast, *IGFBP-3* expression was lower in MDA PCa 2a and MDA PCa 2b cells co-cultured with osteoblasts than in control MDA PCa 2a and MDA PCa 2b cells cultured alone (Fig. 6).

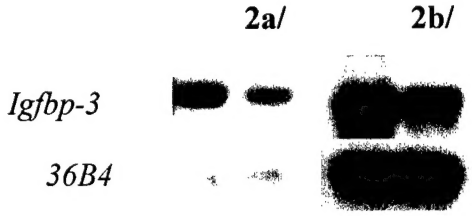



Fig 6. Insulin-like growth factor binding protein 3 (*Igfbp-3*) in MDA PCa 2a (2a) and MDA PCa 2b (2b) cells cultured alone and co-cultured with PMOs (2a/PMO; 2a/PMO) assessed by Northern blotting. *36B4* was used as a loading control.

**These results indicate that IGFBP-3 expression in our *in vitro* model system is modulated at the transcriptional level.** Moreover, IGFBP-3 protein levels would be further decreased in our *in vitro* model system by PSA-mediated proteolysis. These two mechanisms will increase local bioavailable levels of IGF-I. IGF-I concentration in the culture medium is about 1nM (7.5 ng/ml) as assessed by a radioimmunoassay kit (Nichols Institute). In agreement with our results cultures of fetal rat calvaria produce 1 nM/day of IGF-I (46). Moreover previous reports indicate that in cultured rat calvaria, IGF-I induce DNA-synthesis at concentrations of 0.1 to 100 nM and collagen synthesis at concentrations of 0.1 to 30 nM (33). **We therefore propose that alternative and/or additive mechanisms decrease IGFBP-3 expression in prostate cancer. This will increase bioavailable levels of IGFs, which should activate the IGF1R/MAPK pathway. MAPK in turn activates *Cbfa1,* resulting in increased *Osteocalcin* and *Collagen type* I expression and formation of calcified matrix.**

We next decided to study the effect of PMOs in the number of prostate cancer cells after 4 days in our *in vitro* model system. There were always more MDA PCa 2a and MDA PCa 2b cells after 4 days in co-culture with PMOs than when they were grown alone, but the difference did not always reach statistical significance. MDA PCa 2a and MDA PCa 2b cells have population doubling times of 87-93 h and 61-73 h, respectively (1), consistent with the well-recognized slow growth of prostate cancer cells. Moreover, these population doubling times were calculated under optimal growth conditions for these cells (20% FBS with supplements (1)) and are expected to be higher than in our co-culture system ($\alpha$-MEM plus 10% FBS plus 5% BRFF-HPC1 medium). We therefore decided to extend the period of co-culture to 12 days for MDA PCa 2a cells and 6 days for MDA PCa 2b cells. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the number of viable cells after co-culture (47). Control experiments demonstrated that the absorbance was directly proportional to the amount of protein in each well. Moreover, the assay was more accurate than assessing cell numbers because these cells tend to form clumps and so are difficult to count. Fig. 7 shows that the number of MDA PCa 2a and MDA PCa 2b cells increased after 12 and 6 days, respectively, in co-culture with PMOs.

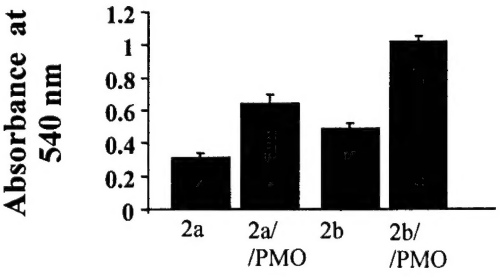



Fig 7. Prostate cancer cell number as assessed by the MTT assay. 2a, MDA PCa 2a cells after 12 days in culture; 2a/PMO, MDA PCa 2a after 12 days in co-culture with PMOs; 2b, MDA PCa 2b after 6 days in culture; 2b/PMO, MDA PCa 2b after 6 days in co-culture with PMOs. Each value represents the mean ± SD of six different experiments. $^{*}p< 0.01$

**These results suggest that PMOs confer a growth or survival advantage on prostate cancer cells.**

Integrin α2ß1 is the major collagen receptor on epithelial cells and platelets (48). The collagen receptor integrins are structurally different from other ECM binding integrins. They have a specific inserted (I) domain as a part of their α subunit (48). This domain is responsible for collagen recognition. We studied expression of α2ß1 integrin complex in prostate cancer cells by fluorescence-activated cell sorter (FACS) analysis. We used the mouse anti-human antibody VLA-2 (α2ß1 clone BHA2.1) (Chemicon International, Inc. Temecula CA) which reacts with the collagen receptor α2ß1 integrin complex. The epitope of BHA2.1 is dependent on the presence of the I domain of α2 integrin subunit and does not bind a VLA-2 variant that lacks the I domain. Fig. 8 shows that the prostate cancer cells tested expressed α2ß1 integrin complex. The expression levels of α2ß1 integrin were 56.2% in MDA PCa 2a, 61.2% in MDA PCa 2b cell, and 98.3% in PC3. These cells are routinely propagated in plastic tissue-culture dishes. α2ß1 integrin expression is probably modulated by the matrix in which they are propagated and may increase in the presence of Collagen.

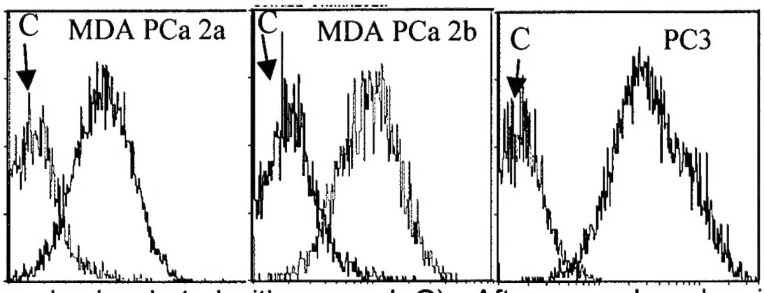



Fig 8. Human prostate cancer cell MDA PCa 2a, MDA PCa 2b, and PC3 cells were harvested with EDTA and resuspended in phosphate-buffered solution (PBS) supplemented with 1mM $MgCl_2$. Primary monoclonal antibody anti-human VLA-2 was added to the cell suspension and incubated for 30 min at room temperature, (as negative control (C) each cell line was also incubated with mouse IgG). After several washes in PBS, the cells were stained with fluoroisothiocyanate (FITC)-labeled anti-mouse secondary antibody (Chemicon) for another 20 min in PBS, washed again with PBS, and analyzed by flow cytometry.

**These results show that prostate cancer cells express the collagen receptor integrin α2ß1**

To confirm *in vivo* the osteoblastic phenotype induced by the MDA PCa 2b cells *in vitro*, we developed an intrabone injection model of bone metastasis. Male SCID mice were obtained from Charles River Laboratories (Wilmington, MA). The animals were anesthetized, and about $1.2 \times 10^6$ MDA PCa 2b or PC3 cells were diluted in 5 μl of growth medium and then injected into the right femurs (or tibia) of 13 mice. The same volume of growth medium was injected into the left femur of each mouse as a control. The mice were then monitored twice weekly for tumor bulk and tested biweekly for PSA serum levels. The animals were killed after 3 months (less if the tumor was bulky) and pathological examination of the subject bones was performed. All mice that had undergone intrabone injections with MDA PCa 2b cells had increased PSA levels. None of the control mice tested had blood PSA levels higher than 0.2 ng/ml. Bone lesions were monitored by X-ray at days 60, 80 and 100. Three mice died before the X-rays were performed. Of the remaining 10 mice, 5, 6, and 8 developed bone lesions (mostly osteoblastic) at days 60, 80, and 100, respectively, after the injection (Fig. 9A). The contralateral legs never showed evidence of osteoblastic lesions. The mice also exhibited features of bone resorption, primarily at the injection site. Nineteen control mice each had $10^6$ PC3 prostate cancer cells injected under the same conditions and were X-rayed

30 to 60 days later. None of the 14 mice that survived the procedure for at least 1 month developed osteoblastic lesions, and 12 (86%) rapidly developed osteolytic lesions (2). Histopathological examination showed evidence of new bone formation when the bones had been injected with MDA PCa 2b. Fig. 9B shows MDA PCa 2b cells growing in the femoral shaft of one injected limb, surrounded by woven (immature) bone, which is typical of rapid bone formation. Surrounding the new bone is the lamellar (mature) bone. In contrast with what occurred with MDA PCa 2b cells, osteoclast recruitment and bone destruction were obvious in bones that had been injected with PC3 cells (2).

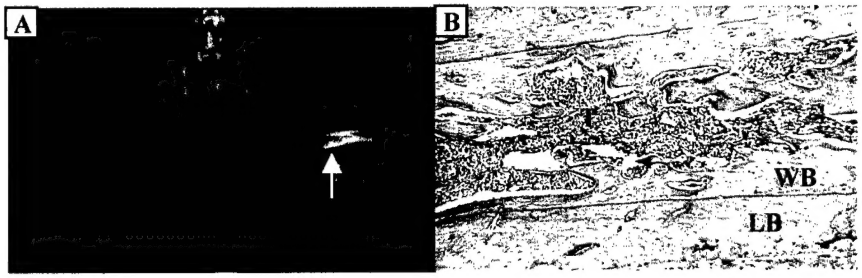



Fig 9. X-ray imaging (A) and microphotography (B) of a mouse femur 10 weeks after intrabone injection of MDA PCa 2b cells. (A) Increased bone density (arrow) after cell injection. (B) Microscopic evidence of new bone formation in the femur of the mice. T, tumor; WB, woven (immature) bone; LB, lamellar (mature) bone. Hematoxylin and eosin staining.

**These results indicated that MDA PCa 2b cells growing in bone, showed evidence of woven bone, which mirror clinical bone metastasis of prostate cancer exhibiting rapid bone formation.**

**Key Research Accomplishments**

- Characterized and optimized *in vitro* and *in vivo* models of prostate cancer bone metastasis.
- Identification of Cbfa-1 dependent pathway as a mediator of the osteoblastic bone metastases.
- Identification of Endothelin-1 dependent and independent mechanisms that concur in the increased bone mass of prostate cancer bone metastases.
- Our data also provide a rationale for developing therapeutic strategies designed to target these molecular changes.
- Development and characterization of an in vivo model of bone metastases that should prove useful for drug screening

**Reportable Outcomes**

1. Characterized and optimized *in vitro* and *in vivo* models of prostate cancer bone metastasis.
2. Published "Prostate cancer cells induce osteoblast differentiation through a Cbfa1-dependent pathway. Yang J, Fizazi K, Peleg S, Sikes CR, Raymond AK, Jamal N, Hu M, Olive M, Martinez LA, Wood CG, Logothetis CJ, Karsenty G, Navone NM. Cancer Res, Jul 15; 61(14): 5652-9, 2001.
3. Manuscript in preparation: Endothelin-1 dependent and independent mechanisms concur in the increased bone mass of prostate cancer bone metastases. Fizazi K, Yang J, Peoel S, Sikes CR, Daliani D, Raymond KA, Janus TJ, Logothetis CJ, Karsenty G, Navone NM.
4. Abstract: 2000 AACR Annual Meeting, San Francisco, CA. Poster presentation – Osteoblast-prostate cancer cells interaction attenuates the antiproliferative effect of vitamin D3. Navone NM, Yang J, Olive M, Logothetis CJ, and Peleg S.
5. Abstract: 2001 AACR Annual Meeting, New Orleans, LA. "p21 and p16 potentiate p53 mediated apoptosis in prostate cancer cells." Luis A. Martinez, June Yang, Karim Fizazi and Nora M. Navone.
6. Abstract: 2001 AACR Annual Meeting, New Orleans, LA. "In Vitro Study of the biological basis of osteoblastic/osteoclastic bone metastases from prostate cancer." Jun Yang, Karim Fizazi, Charles Sikes, Luis A. Martinez, Kevin Raymond, Christopher Logothetis, Sara Peleg, and Nora M. Navone.
7. Personnel: Charles R. Sikes
8. Abstract: 2001 AACR Annual Meeting, New Orleans, LA. "p53 regulates the response to growth factor deprivationa dn androgen withdrawal in prostate cancer cells in vitro and in vivo. Nora M. Navone, Jun Yang, Karim Fizazi, Charles Sikes, Elba Vazquez, and Luis A. Martinez.

9. Abstract: 2001 AACR Annual Meeting, New Orleans, LA. "The effects of endothelin-1 and Abt-627, an endothelin-1 antagonist, in an in vitro model of bone metastases from prostate cancer. Karim Fizazi, Jun Yang, Danai Daliani, Christopher Logothetis, Sara Peleg, and Nora M. Navone.
10. Presentation: 1999 CaP CURE Annual Scientific Retreat – "Rational Basis for Bone-Homing Therapies for Human Prostate Cancer"
11. Funded grant: 1999-2000 CaP CURE Foundation. "Rational Basis for Bone-Homing Therapies for Human Prostate Cancer." Principal Investigator: Nora M. Navone. Total Award: $50,000.
12. Funded grant: 2000-2001 CaP CURE Foundation. "Metastatic Prostate Cancer: Integration of Bone and Prostate Cancer Cell Interaction." Principal Investigator: Nora M. Navone. Total Award: $100,000

**Conclusions**

Bone metastasis in prostate cancer are characteristically blastic, the first site of androgen independent progression, and account for a large portion of the clinical morbidity. The importance of bone metastasis in prostate cancer is reflected by the remarkable affinity of prostate cancer to develop bone metastasis as the only site of progression. This strongly suggests that the interaction of malignant epithelial cells with the bone microenvironment actively contributes to the lethal progression of prostate cancer. Understanding the factors that determine or influence the tropism of prostate cancer cells to bone may lead to treatments that prevent or revert prostate cancer metastases. One of the major obstacles to understanding the androgen-independent growth of prostate cancer cells within bone has been the lack of functionally relevant model systems of advanced prostate cancer. We have established and characterized in vitro and an in vivo models of bone metastases. These models are essential to decipher the molecular events of androgen-independent growth of prostate cancer within to decipher the molecular events of androgen-independent growth of prostate cancer within bone. Using the in vitro model we have shown that factors produced by the bone-derived prostate cancer MDA PCa 2a and MDA PCa 2b cells increased expression of markers of osteoblast differentiation and calcified matrix formation. Our studies also indicated that endothelin-1, which has been implicated in the osteoblastic response produced by prostate cancer bone metastases, is only partially responsible for that effect. Our preliminary results also implicated the IGFs pathway in the pathophysiology of prostate cancer bone metastasis. In summary, our results provide evidence that multiple and distinct molecular events affecting bone formation concur and result in an increase in bone mass. Our data also provide a rationale for developing therapeutic strategies designed to target these molecular changes.

**8.0 References**


1. Navone NM, Olive M, Ozen M, Davis R, Troncoso P, Tu S-M, Johnson D, Pollack A, Pathak S, von Eschenbach AC, Logothetis CJ. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. *Clin Cancer Res* 3: 2493-2500, 1997.
2. Yang J, Fizazi K, Peleg S, Sikes CR, Raymond AK, Jamal N, Hu M, Olive M, Martinez LA, Wood CG, Logothetis CJ, Karsenty G, Navone NM. Prostate cancer cells induce osteoblast differentiation through a Cbfa1-dependent pathway. *Cancer Res* Jul 15;61(14):5652-9, 2001.
3. Navone NM, Logothetis CJ, von Eschenbach AC, Troncoso P. Model systems of prostate cancer: uses and limitations. *Cancer Met Rev* 17: 361-371, 1999.
4. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 89: 747-54, 1997.
5. Karsenty G. The genetic transformation of bone biology. *Genes Dev* 13(23): 3037-51, 1999.
6. Kern B, Shen J, Starbuck M, Karsenty G. Cbfa1 contributes to the osteoblast specific expression of type I collagen genes. *J Biol Chem* 276(10): 101-107, 2001.
7. Bellows CG, Aubin JE, Heersche JNM, Antosz ME. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* 38: 143-154, 1986.
8. Bellows CG, Aubin JE. Determination of numbers of osteoprogenitors present in isolated fetal rat calvaria cells in vitro. *Dev Biol* 133: 8-13, 1989.
9. Malaval L, Liu F, Roche P, Aubin JE. Kinetics of osteoprogenitor proliferation and osteoblast differentiation in vitro. *J Cell Biochem* 74: 616-627, 1999.
10. Thomas GP, Bourne A, Eisman JA, Gardiner EM. Species-divergent regulation of human and mouse osteocalcin genes by calciotropic hormones. *Exp Cell Res* 258: 395-402, 2000.

11. Mansukhani A, Bellosta P, Sahni M, Basilico C. Signaling by fibroblast growth factor (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induced apoptosis in osteoblasts. *J Cell Biol* 149: 1297-1308, 2000.
12. Bharagava U, Bar-Lev M, Bellows CG, Aubin JE. Ultrastructural analysis of bone nodules formed in vitro by isolated fetal rat calvaria cells. *Bone* 9:155-163, 1986.
13. Martinez J, Silva S, Santibanez JF. Prostate-derived soluble factors block osteoblast differentiation in culture. *J Cell Biochem* 61: 18-25, 1996.
14. Kido J-I, Yamauchi N, Ohishi K, Kataoka M, Nishikawa S, Nakamura T, Kadono H, Ikedo D, Ueno A, Nonomura N, Okuyama A, Nagata T. Inhibition of osteoblast cell differentiation by conditioned medium derived from the human prostatic cancer cell line PC-3 in vitro. *J Cell Biochem* 67: 248-256, 1997.
15. Yamaguchi A, Komori T, and Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocr Rev* 21(4): 393-411, 2000.
16. Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G. Missense mutation abolishing DNA binding of the osteoblast-specific transcriptor factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet* 16: 307-10, 1997.
17. Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev* 13: 1025-1036, 1999.
18. Ducy P. *Cbfa1*: A molecular switch in osteoblast biology. *Dev Dyn* 219:461-471, 2000.
19. Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem* 275: 4453–4459, 2000.
20. Killian CS, Corral DA, Kawinski E, and Constantine RI. Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent TGF-β and a proteolytic modulation of cell adhesion receptors. *Biochem Biophys Res Commun* 192: 940-947, 1993.
21. Cramer SD, Chen Z, and Peehl DM. Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTH-rP stimulated cAMP accumulation in mouse osteoblasts. *J Urol* 156: 526-531, 1996.
22. Achbarou A, Kaiser S, Tremblay G, Ste-Marie LG, Brodt P, Goltzman D, and Rabbani SA. Urokinase overproduction results in increased skeletal metastasis by prostate cancer cells in vivo. *Cancer Res* 54: 2372-2377, 1994.
23. Bentley H, Hamdy FC, Hart KA, Seid JM, Williams JL, Johnstone D, and Russell RGG. Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia. *Br J Cancer* 66: 1159-1163, 1992.
24. Harris SE, Harris MA, Mahy P, Wozney J, Feng JQ, and Mundy GR. Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells. *Prostate* 24: 204-211, 1994.
25. Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA, and Simons JW. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med* 1: 944-949, 1995.
26. Boyce BF, Yoneda T, Guise TA. Factors regulating the growth of metastatic cancer in bone. *Endocr Relat Cancer.* 6: 333-347, 1999.
27. Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA, Simons JW. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med* 1: 944-949, 1995.
28. Kasperk CH, Borcsok I, Schairer HU, Schneider U, Nawroth PP, Niethard FU, Ziegler R. Endothelin-1 is a potent regulator of human bone cell metabolism in vitro. *Calcif Tissue Int.* 60: 368-374, 1997.
29. Nelson JB, Nguyen SH, Wu-Wong JR, Opgenorth TJ, Dixon DB, Chung LW, Inoue N. New bone formation in an osteoblastic tumor model is increased by endothelin-overexpression and decreased by endothelin A receptor blockade. *Urology* 53: 1063-1069, 1999.
30. Pietrzkowski Z, Mulholland G, Gomella L, Jameson BA, Wernicke D, and Baserga R. Inhibition of growth of prostatic cancer cell lines by peptide analogues of insulin-like growth factor 1. *Cancer Res* 53: 1102-1106, 1993.

31. Ritchie CK, Andrews LR, Thomas KG, Tindall DJ, and Fitzpatrick LA. The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis: implications for the development of metastatic disease. *Endocrinology*. 138: 1145-1150, 1997.
32. Conover C. The role of insulin-like growth factors and binding proteins in bone cell biology. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, New York, pp 607–618, 1996.
33. Canalis E. Effects of insulin-like growth factor I on DNA and protein synthesis in culture rat calvaria. *J Clin Invest* 66:709 –719, 1980.
34. Jia D, Heersche JNM. Insulin-like growth factor-1 and -2 stimulate osteoprogenitor proliferation and differentiation and adipocyte formation in cell populations derived from adult rat bone. *Bone* 27, 785–794, 2000.
35. Grimberg A, Cohen P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cellular Phys* 183:1–9 2000.
36. McCarthy TL, Centrella M, Canalis E. Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures. *Endocrinology* 124:301–309, 1989.
37. Zhao G, Monier-Faugere M-C, Langub MC, Geng Z, Nakayama T, Pike JW, Chernausek SD, Rosen CJ, Donahue L-H, Malluche HH, Fagin JA, Clemens TL. Targeted overexpression of insulin-like growth factor I to osteoblasts of transgenic mice: increased trabecular bone volume without increased osteoblast proliferation. *Endocrinology* 141: 2674-82, 2000.
38. Liu J-P, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75:59–72, 1993.
39. Baserga R. The contradictions of the insulin-like growth factor 1 receptor. *Oncogene* 19, 5574-5581, 2000.
40. Yu H, and Rohan T. Role of the insulin-like growth factor family in cancer development and progression. *J Natl Cancer Inst* 92: 1472-1489, 2000.
41. Ballard FJ, Knowles SE, Walton PE, Edson K, Owens PC, Mohler MA, Ferraiolo BL. Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I), IGF-II and des(1-3)IGF-I in rats. *J Endocrinol* 128(2):197-204, 1991.
42. Koutsilieris M, Mitsiades C, Sourla A. Insulin-like growth factor I and urokinase-type plasminogen activator bioregulation system as a survival mechanism of prostate cancer cells in osteoblastic metastases: development of anti-survival factor therapy for hormone-refractory prostate cancer. *Mol Med* 6: 251-267, 2000.
43. Kanety H, Madjar Y, Dagan Y, Levi J, Papa MZ, Pariente C, Goldwasser B, Karasik A. Serum insulin-like growth factor-binding protein-2 (IGFBP-2) is increased and IGFBP-3 is decreased in patients with prostate cancer: correlation with serum prostate-specific antigen. *J Clin Endocrinol Metab* 77(1):229-33, 1993.
44. Smith GL, Doherty AP, Mitchell H, Hanham IW, Christmas TJ, Epstein RJ. Inverse relation between prostate-specific antigen and insulin-like growth factor-binding protein 3 in bone metastases and serum of patients with prostate cancer. *Lancet* 354:2054-2055, 1999.
45. Doherty AP, Smith GL, Banks L, Christmas TJ, Epstein RJ. Correlation of the osteoblastic phenotype with prostate-specific antigen expression in metastatic prostate cancer: implications for paracrine growth. *J Pathol* 188: 278-281, 1999.
46. Canalis E, McCarthy TL, Centrella M. Isolation and characterization of insulin-like growth factor I (Somatomedin-C) from cultures of fetal rat calvariae. *Endocrinology* 122:22–27, 1988.
47. Plumb JA, Milory R, Kaye SB. Effects of the pH dependence of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetra-zolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res* 49: 4435-4440, 1989.

[CANCER RESEARCH 61, 5652–5659, July 15, 2001]

# Prostate Cancer Cells Induce Osteoblast Differentiation through a *Cbfa1*-dependent Pathway[1]

**Jun Yang,[2] Karim Fizazi,[2] Sara Peleg, Charles R. Sikes, A. Kevin Raymond, Nausheen Jamal, Min Hu, Matilde Olive, Luis A. Martinez, Christopher G. Wood, Christopher J. Logothetis, Gerard Karsenty, and Nora M. Navone[3]**

*Department of Genitourinary Medical Oncology [J. Y., K. F., C. R. S., M. O., L. A. M., C. J. L., N. M. N.], Pathology [A. K. R.], Urology [C. G. W.], and Endocrine Neoplasia and Hormonal Disorders [S. P., N. J., M. H.], The University of Texas M. D. Anderson Cancer Center, and Department of Molecular and Human Genetics Baylor College of Medicine [G. K.], Houston, Texas 77030*

## ABSTRACT

**Metastases from prostatic adenocarcinoma (prostate cancer) are characterized by their predilection for bone and typical osteoblastic features. An *in vitro* model of bone metastases from prostate cancer was developed using a bicompartment coculture system of mouse osteoblasts and human prostate cancer cells. In this model, the bone-derived prostate cancer cell lines MDA PCa 2a and MDA PCa 2b induced a specific and reproducible increase in osteoblast proliferation. Moreover, these cells were able to induce osteoblast differentiation, as assessed by increased alkaline phosphatase activity, *Osteocalcin* expression, and calcified matrix formation. This osteoblastic reaction was confirmed *in vivo* by intrafemoral injection of MDA PCa 2b cells into severe combined immunodeficiency disease mice. In contrast, the highly undifferentiated, bone-derived human prostate cancer cell line PC3 did not produce an osteoblastic reaction *in vitro* and induced osteolytic lesions *in vivo*. The osteoblast differentiation induced by MDA PCa 2b cells was associated with up-regulation of the osteoblast-specific transcriptor factor *Cbfa1*. Moreover, treatment of osteoblasts with conditioned medium obtained from MDA PCa 2b cells resulted in up-regulation of *Cbfa1* and *Osteocalcin* expression. In support of the differentiation studies, a microarray analysis showed that primary mouse osteoblasts grown in the presence of MDA PCa 2b cells showed a shift in the pattern of gene expression with an increase in mRNA-encoding *Procollagen type I* and *Osteopontin* and a decrease in mRNA-encoding proteins associated with myoblast differentiation, namely myoglobin and myosin light-chain 2. Taken together, these findings suggest that the bone-derived prostate cancer cells MDA PCa 2a and MDA PCa 2b promote differentiation of osteoblast precursors to an osteoblastic phenotype through a *Cbfa1*-dependent pathway. These results also established that soluble factors produced by prostate cancer cells can induce expression of osteoblast-specific genes. This *in vitro* model provides a valuable system to isolate molecules secreted by prostate cancer cells that favor osteoblast differentiation. Moreover, it allows to screen for therapeutic agents blocking the osteoblast response to prostate cancer.**

## INTRODUCTION

Prostate cancer is currently recognized as the most frequent form of cancer in males and the second leading cause of cancer death in men in the United States (1). Although localized prostate cancer may be cured, 70% of patients with metastases will die of cancer rather than an unrelated cause (2, 3). Characteristically, the metastatic dissemination pattern of prostate cancer is: (*a*) the spread has a predilection for bone in ~80% of cases; and (*b*) bone metastases are typically osteoblastic (4). Usually bone metastases are initially sensitive to testosterone deprivation, but given sufficient time, androgen-independent growth eventually occurs in all cases. This latter situation is associated with bone complications and carries a poor prognosis, with median survival of <1 year. This strongly suggests that the interaction of prostatic cancer cells with cells of the osteoblast lineage contributes to the lethal progression of prostate cancer, although the molecular nature of this interaction is still poorly understood. In particular, it is not known whether this interaction requires cell-to-cell contact. The classical "seed-and-soil" hypothesis proposes that neoplastic cells prefer to colonize an organ that may serve as fertile soil (5). One interpretation of this hypothesis is that prostate cancer cells may be specifically attracted by factors released from bone and, thus, migrate preferentially to it (6). Another plausible explanation involves the osteomimetic properties of prostatic metastases to support their predilection to bone (7).

One major hindrance in the study of the biology of metastatic prostate cancer has been the limited number of laboratory models of prostate cancer, compared with the number of models available for other neoplasms (8, 9). Indeed, no reliable *in vitro* model of osteoblastic bone metastasis from prostate cancer is currently available. Thus, establishing a reliable model of bone metastases would be important. This would allow us to have a better understanding of its biology, to study at the molecular level the interaction of prostate cancer cells with osteoblast, to develop more efficient therapies (especially bone-targeted therapies), and to further investigate the mechanisms of resistance to drugs in bone.

We previously established two prostate cancer cell lines: MDA PCa 2a and MDA PCa 2b (10, 11). These cell lines are the first ones derived from a bone metastasis of prostate cancer to possess typical features of prostate cancer, because they express PSA,[4] the androgen receptor, and their proliferation is regulated by androgens. In this study, we cocultured MDA PCa 2a and MDA PCa 2b with mouse osteoblasts. We established and optimized this *in vitro* model of bone metastases from prostate cancer and showed evidence that these cells induced a specific increase in osteoblast growth and differentiation. We also demonstrated that these biological events are associated with an increase in expression of *Cbfa1, Procollagen type I, Osteocalcin,* and *Osteopontin*, whereas the expression of genes associated with myoblast differentiation was repressed.

## MATERIALS AND METHODS

**Cell Culture.** MDA PCA 2a and MDA PCA 2b cell lines (11) were routinely propagated in BRFF-HPC1 medium (Biological Research Faculty and Facility, Inc., Jamsville, MD) with 20% FBS (Life Technologies, Inc., Gaithersburg, MD). LNCaP, PC3, HeLa, T24, ROS-17/2.8, and CV-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD). LNCaP was maintained in RPMI 1640, ROS-17/2.8 was maintained in DMEM/F12K, the other cell lines were maintained in DMEM (Life Technologies, Inc.), and all were supplemented with 10% FBS. The immortalized

Received 3/1/01; accepted 5/31/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by CaP CURE, DAMD17-991-9028, NIH Grant CA75499-04, DK 50583, and the Ligue contre le cancer.

[2] J. Y. and K. F. contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of GU Medical Oncology, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 013, Houston TX 77030. Phone: (713) 792-2898; Fax: (713) 792-5696; E-mail: nnavone@mail.mdanderson.org.

[4] The abbreviations used are: PSA, prostate-specific antigen; FBS, fetal bovine serum; PMO, primary mouse osteoblast; CM, conditioned medium; α-MEM, α-modified Eagle medium.

mouse preosteoblast cell line 2T3 (12) was a generous gift from Stephen Harris, University of Texas Health Science Center, San Antonio, TX. These cells were grown in α-MEM (Life Technologies, Inc.) plus 10% FBS.

**Primary Cultures of Mouse Calvaria Osteoblasts.** To obtain primary culture of osteoblasts, we used newborn CD1 mice that were killed 4 days after birth. Cultures of PMOs were obtained from the calvaria by use of a procedure published previously (13), with slight modifications. In brief, after dissection, calvaria were digested for 15 min in a shaking incubator at 37°C in 15 ml of α-MEM containing 0.1 mg/ml collagenase P (Boehringer Mannheim, Corp, Indianapolis, IN), 2.5% trypsin/EDTA (Life Technologies, Inc.), streptomycin, and penicillin. The mixtures were also gently shaken by hand for 20 s every 5 min during the procedure. The digestion medium and any released cells were then discarded. This entire procedure was repeated for another 15 min, and the medium was discarded again. Finally, it was repeated again for 25 min, and the cell suspensions were transferred to new tubes and washed with α-MEM plus 10% FBS. This last procedure was repeated three times; each time, the cell suspensions were collected and pooled. The cells were then plated in α-MEM plus 10% FBS for 48 h. The cells were then subsequently trypsinized and replated in culture dishes to perform experiments.

**Cbfa1 Transcriptional Activity.** To examine transcriptional activity mediated by Cbfa1, we transfected PMOs with a reporter gene containing the multimerized *Cbfa1* response element (OSE2; 5′-GATCCGCTGCAATCAC-CAACCACAGCA-3′; Ref. 14) inserted upstream from the minimal promoter of the firefly luciferase expression vector pTAL-Luc (Promega, Corp., Madison, WI). The cells were plated at a density of 5,000 cells/cm$^2$ in 35-mm dishes in α-MEM containing 10% FBS 24 h before transfection. On the day of transfection, the medium was changed to serum-free α-MEM, and the cells were treated with a 3:1 mixture of Lipofectamine reagent (Life Technologies, Inc.) and DNA for 5 h, at which time FBS was added to a final concentration of 10%. The medium containing the transfection reagent was removed after 24 h and replaced with fresh medium containing α-MEM and 10% FBS. For measurements of luciferase activity, the transfected cells were lysed 72–96 h after transfection with CCLR lysis buffer (Promega, Corp.) and assayed with the luciferase assay reagent, according to the manufacturer's instructions (Promega, Corp.). Light units were measured with a luminometer (TD 20/20; Turner Designs Inc., Sunnyvale, CA) and normalized for protein concentrations in each cell lysate. Each transfection experiment was performed in triplicate, with pTAL-Luc as a control, to determine the up-regulation of the minimal promoter activity by the multimerized OSE2 enhancer (5xOSE2-pTAL construct). To examine the cell specificity of the OSE2-mediated transcription, we performed similar transfection experiments with the rat osteosarcoma cell line ROS-17/2.8 (positive control) and the monkey kidney cell line CV-1.

**Coculture of PMOs with Human Cancer Cells.** An *in vitro* bicompartment culture system was developed as a model of bone metastases from prostate cancer. PMOs were seeded in tissue culture plates, whereas MDA PCa 2a or MDA PCa 2b were seeded in cell-culture inserts (0.4-μm pore; Falcon/Becton Dickinson Labware, Franklin Lakes, NJ). Coculturing was performed with α-MEM plus 5% BRFF-HPC1 as a growth medium for all cell types used in the inserts. A similar method was used to coculture LNCaP, PC3, HeLa, and T24 with PMOs. LNCaP is derived from a lymph node metastasis, not from a bone metastasis. PC3 is derived from a bone metastasis but does not produce PSA and is not regulated by androgens. HeLa and T24, two neoplastic cell lines not from prostate origin, were used as additional controls. We optimized this coculture model for the optimal number of cells to be seeded so that the cells would reach 80% confluence after 4 days of culturing. The optimal numbers of cells selected were as follows: (*a*) MDA PCa 2a or MDA PCa 2b, 20,000 cells/cm$^2$; (*b*) LNCaP, 10,000 cells/cm$^2$; (*c*) PC3, 1,000 cells/cm$^2$; (*d*) HeLa, 1,000 cells/cm$^2$; (*e*) T24, 750 cells/cm$^2$; and (*f*) PMO, 5,000 cells/cm$^2$. After 24 h of cell culturing, the inserts were placed into tissue-culture plates containing the PMOs so that the two different cell types shared the culture medium but were not in physical contact. Each experiment was assayed six times. The medium was changed every 2 days.

To assess if prostate cancer cells affected the ability of PMOs to differentiate after PMOs and prostate cancer cells had been separated, PMOs were cocultured in our system both without (control) and with MDA PCa 2a, MDA PCa 2b, LNCaP, and PC3 cells. After 4 days of coculturing, the inserts containing the prostate cancer cells were removed, and the PMOs were maintained in culture until they reached confluence (day 0). The PMOs were subsequently placed in differentiation medium (α-MEM plus 10% FBS, 100 μg/ml ascorbic acid, and 10 nM sodium β-glycerophosphate) in the presence of BMP-2 (20 ng/ml). PMOs growing in these conditions were tested for alkaline phosphatase activity every 2 days, from days 4 to 12. Calcified matrix formation was assessed by von Kossa staining at day 16 of culture in differentiation medium. Each experiment was performed in duplicates.

**Coculture of Immortalized Murine Osteoblasts (2T3 Cells) with Prostate Cancer Cells.** We tested the reproducibility of our findings by using a well-characterized clonal osteoblast cell line, 2T3, derived from mouse calvaria (12). PMOs were replaced in the model by the 2T3 cells. In these experiments, 1000 cells/cm$^2$ 2T3 cells were cocultured with MDA PCa 2a, MDA PCa 2b, or LNCaP cells under the conditions already described. After 4 days of coculturing, the number of 2T3 cells was assessed. As described for PMOs, the 2T3 cells were then grown in differentiation medium for 8 days, and alkaline phosphatase activity and osteocalcin production were assessed. Each experiment was performed in triplicates.

**Alkaline Phosphatase Activity and Osteocalcin Secretion Level.** Alkaline phosphatase activity was determined in cell extracts with the Sigma Chemical Co. Diagnostics alkaline phosphatase reagent (St. Louis, MO). The level of osteocalcin in the culture medium was determined using a mouse osteocalcin immunoradiometric assay kit (Immunotopics, Inc., San Clemente, CA).

**Mineralized Bone Matrix Formation Assay.** Bone cell differentiation was monitored by using an assay for mineralized matrix formation (15), with slight modifications. In brief, von Kossa's staining of mineralized bone matrix was performed as follows: cell cultures were washed twice with PBS, fixed in phosphate-buffered formalin for 10 min, and washed with water. Remaining water was removed, a 5% silver nitrate solution was added, and plates were incubated in bright light for 20 min. The reaction was stopped by rinsing the plates in water. Finally, 5% sodium thiosulfate was added, and the plates were rinsed twice with water.

**CM Preparation and PMO Treatment.** To assess the effect of soluble factors produced by MDA PCa 2b cells on gene expression of PMOs, we obtained CM from MDA PCa 2b cells. MDA PCa 2b cells were grown in 100-mm tissue culture dishes until 60–70% confluent. We subsequently washed them with PBS and changed the medium to serum-free α-MEM. After 48 h, the CM was collected and filtered through a 0.2-μm sterilizing filter. This crude CM was used as PMO growth medium. After 6 h, PMOs were collected, the RNA was isolated, and a Northern blot analysis was performed.

The CM prepared as described was also concentrated by ammonium sulfate precipitation: 70 g of ammonium sulfate were dissolved in 100 ml of CM, the procedure was performed at 4°C, and the solution was stirred until dissolution was complete. The solution was then centrifuged at 10,000 g × 45 min, the supernatant was discarded, and the pellet (dissolved in a minimal volume) was dialyzed overnight at 4°C against 0.005 M 4-morpholinepropanesulfonic acid with 500-kDa cutoff membrane. The dialyzed product was recovered in a volume of 2.4 ml (~40-fold concentration of the original CM); we refer to this medium as $CM_{70}$.

PMOs were then grown in α-MEM plus $CM_{70}$, supplemented with 10% FBS. $CM_{70}$ was added to the growth medium to produce a final concentration of three times the crude CM (3 × $CM_{70}$). After 12 h, PMOs were collected, RNA was isolated, and a Northern blot analysis was performed.

**RNA Extraction and Northern Blot Analysis.** Total RNA was extracted from PMO cell monolayers by using the RNAzol B Reagent (Biotecx Laboratories, Inc. Houston, TX). Then the RNA (20 μg) was denatured, and electrophoresis was conducted in sample buffer (50% formamide, 10% 4-morpholinepropanesulfonic acid buffer, 18% formaldehyde, 10% glycerol, 0.5% bromphenol blue, and 1 μg of ethidium bromide) on agarose-formaldehyde gel (80 V, 3.5 h). Finally, the RNA was transferred to nylon membranes (Bio-Rad Laboratories, Hercules CA) via passive transfer overnight and then cross-linked by UV transillumination. The membranes were prehybridized at 65°C for 3 h in a Rapid-Hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ) plus 1 mg/ml denatured salmon sperm DNA. Specific cDNA probes were purified and labeled with [$\alpha^{32}$]P dCTP with a random primer labeling kit (Megaprime DNA Labeling System; Amersham Pharmacia Biotech). Hybridization with the probes *Myoglobin* (clone ID #738009), *Myosin light chain 2* (clone ID #466382; Incyte Genomics, Palo Alto, CA), *Cbfa1, Osteocalcin, Osteopontin*, and *Procollagen, type I α 1* (16) was carried out overnight at 65°C. The membranes were washed three times at the same temperature with decreasing concentrations of SSC (2×, 0.5×, and 0.1×) in 0.5% SDS. The blots were then exposed to Kodak X-OMAT film at −80°C and developed in

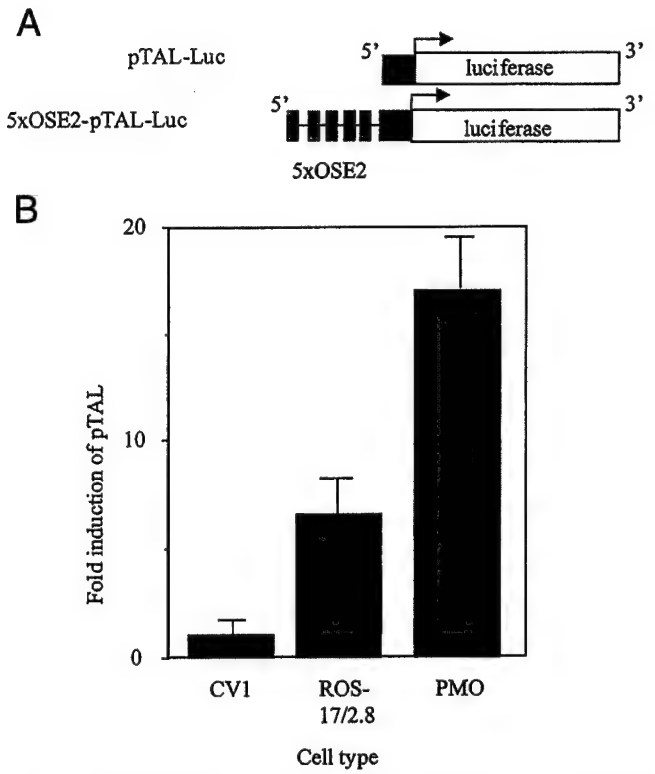



Fig. 1. Transcriptional activity of *Cbfa1* in PMOs. *A*, PMO, ROS 17/2.8, and CV-1 cells were transfected with the reporter 5xOSE2-pTAL-Luc or its control (pTAL-Luc) *B*, and reporter gene expression was measured 72 h later. Results are expressed as fold induction of pTAL-Luc activity by the OSE2 enhancer. Each transfection experiment was performed in triplicate. The transfection experiments with PMO or with ROS 17/2.8 cells were performed four times and were also performed twice with CV-1 cells.

an automatic film processor. Blots were also stripped and reprobed with a cDNA probe for 36B4 (17) or *Gapdh* as a control for even loading.

**Gene Array Analysis.** The differential expression of multiple genes in PMOs grown alone compared with that in PMOs grown in the presence of MDA PCa 2b cells was analyzed with the GEM 1 Gene Expression Microarray (Incyte Genomics). RNA was isolated from PMOs grown either alone (control) or cocultured with MDA PCa 2b. Poly(A)+ RNA preparation, cDNA probe synthesis, hybridization with Mouse GEM 1 cDNA microarray, and signal analysis were conducted by Incyte Genomics.[5]

**Intrabone Injections.** An intrabone injection model of bone metastasis was developed to confirm *in vivo* the osteoblastic phenotype induced by the MDA PCa 2b cells *in vitro*. Male severe combined immunodeficiency disease mice obtained from Charles River Breeding Laboratories (Wilmington, MA) were housed in a facility with constant humidity and temperature and a 12-h light-dark cycle. They had *ad libitum* access to standard mouse feed and water and were monitored daily. Animals were anesthetized with i.m. injections of ketamine 100 mg/kg plus acepromazine 2.5 mg/kg. An average number of $1.2 \times 10^6$ of MDA PCa 2b or PC3 cells was diluted in 5 $\mu$l of growth medium, and then 13 mice were injected into the right femur (or tibia) of each mouse. The same volume of growth medium was injected into the left femur of each mouse as a control. Mice were then monitored twice weekly for tumor bulk and tested biweekly for PSA serum levels. Radiographs of the bones that had received the injections were obtained every month and before the mice were killed. Animals were killed after 3 months (less in case of a bulky tumor), and a pathological examination of the subject bones was performed.

**PSA Blood Levels.** Blood from the mice was obtained at regular intervals from a small incision in the main tail vein. Serum was separated from the blood, and PSA was measured using a microparticle enzyme immunoassay (IMx PSA assay, Abbott Laboratories, Abbott Park, IL).

**Tissue Samples.** Formalin-fixed, paraffin-embedded tissue samples from the tumors were prepared by the Department of Veterinary Medicine of M. D. Anderson Cancer Center. The subject bones were dissected free of muscle, fixed in 10% buffered formalin, decalcified in 5% formic acid, and then embedded in paraffin. Longitudinal 3-$\mu$m-thick sections were obtained from each sample and stained with H&E.

**Statistical Analysis.** Numeric data were expressed as means ± SE. Statistical differences between means for the different groups were evaluated with Sigma Chemical Co. Plot 4 one-way ANOVA and Tukey's mean separation test, with the level of significance set at $P < 0.05$.

## RESULTS

**PMOs Express Active Cbfa1 Transcription Factor.** We assessed if *Cbfa1* expressed in PMOs was transcriptionally active. *Cbfa1* is a gene encoding a transcription factor whose function is critical for osteoblast differentiation, and it is the earliest known molecular marker of osteogenesis. *Cbfa1* is expressed in cells destined to become osteoblasts but not in any other cells at any significant level (18). We found previously that *Cbfa1* RNA is expressed in PMOs (19). Fig. 1*B* shows that the *Cbfa1* response element (OSE2) increased the minimal promoter activity of the reporter pTAL by 15- to 20-fold in PMOs and 5- to 6-fold in the osteoblast-like ROS-17/2.8 cells. In contrast, there was no detectable activity in the CV-1 kidney cell line. These results confirm the cell specificity of OSE2-mediated transcription and suggest that the cells isolated from calvaria contain a high

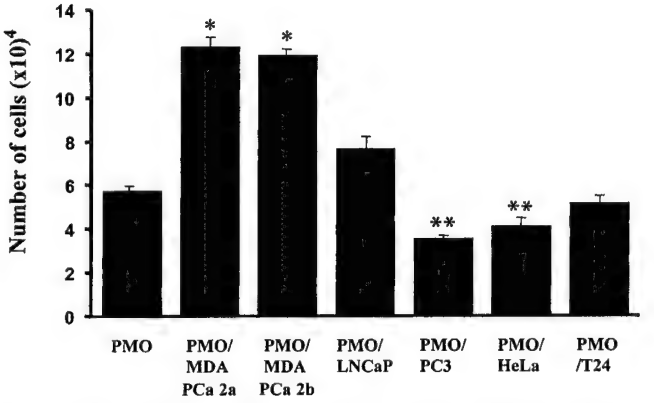



Fig. 2. Number of cells in PMO cultures grown for 4 days either alone (*PMO*) or in coculture with MDA PCa 2a, MDA PCa 2b, LNCaP, PC3, HeLa, and T24 cells. Each culture or coculture was assayed six times. Cells were counted in a hematocytometer. Results obtained from three different experiments are expressed as mean; *bars*, SE. *, cell number of cocultured PMOs significantly higher than controls ($P < 0.001$). **, cell number of cocultured PMOs significantly lower than controls ($P < 0.001$).

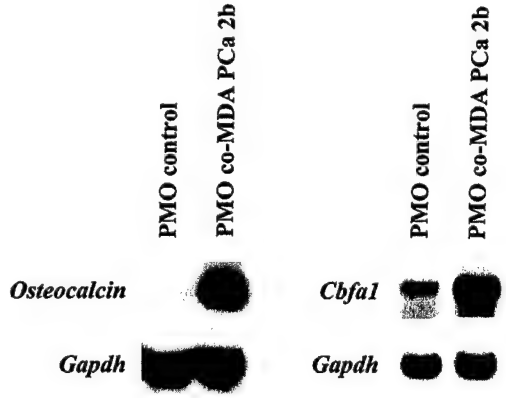



Fig. 3. Northern blot analysis of *Cbfa1* and *Osteocalcin* expression in PMOs grown alone (*control*) or cocultured with MDA PCa 2b cells. *Gapdh* was used as a loading control.

[5] Internet address: http://www.incyte.com.

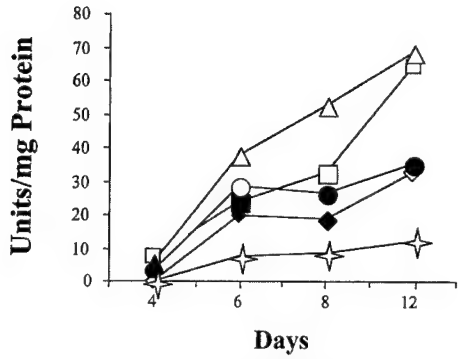



Fig. 4. Alkaline phosphatase activity in PMOs. PMOs were grown either alone (♦) or in coculture with MDA PCa 2a (□), MDA PCa 2b (△), LNCaP (●), and PC3 (✧). They were separated after 4 days of coculture. PMOs were subsequently grown in $\alpha$-MEM plus 10% FBS until they reached confluence, and then they were placed in differentiation medium containing BMP-2. Alkaline phosphatase activity (units/mg protein) was measured at the indicated days using a Sigma Chemical Co. Diagnostics kit.

amount of transcriptionally active *Cbfa1,* and therefore, they are likely to include osteoblasts or osteoblast precursors.

**Specificity of PMO Proliferation Induced by Coculturing with MDA PCa 2a and MDA PCa 2b Cells.** Evidence of a significant increase in PMO proliferation was found when these cells were cocultured with MDA PCa 2a and MDA PCa 2b cells ($P < 0.001$ for both, Fig. 2). This increase was confirmed in four consecutive experiments and was always consistent. When PMOs were cocultured with LNCaP cells, a proliferative response was also found in the PMOs, although the magnitude of this response varied between experiments. In contrast, when PMOs were cocultured with PC3 cells, the number of PMOs decreased significantly ($P < 0.001$). Moreover, the number of PMOs also decreased significantly after coculturing with HeLa cells ($P < 0.001$), whereas no significant modification was observed with T24 cells (Fig. 2). These results suggest that the proliferative response observed with the MDA PCa 2a and MDA PCa 2b bone-derived prostate cancer cells is not universal and may be specific.

**Coculturing with Prostate Cancer Cells Promotes Differentiation of PMOs.** Cells isolated from calvaria comprise osteoblasts at multiple stages of differentiation. In prolonged culture, these cells undergo a defined series of events from proliferation to maturation, express *Osteopontin, Alkaline phosphatase, Bone sialoprotein,* and *Osteocalcin* genes, and ultimately form mineral (20–24). Of those four genes, only the *Osteocalcin* genes are expressed solely in osteoblasts and no other extracellular matrix-producing cell (18). To assess if the prostate cancer cells could induce differentiation of osteoblast cells, we studied *Osteocalcin* expression in the PMOs grown alone and 4 days of coculturing with MDA PCa 2b. As Fig. 3 shows, *Osteocalcin* expression was increased in PMOs that had been grown in the presence of MDA PCa 2b cells compared with PMOs grown alone. *Cbfa1* is a central regulator of osteoblast differentiation and function and, as such, activates most of the genes expressed by osteoblasts (18). Fig. 3 also shows that *Cbfa1* expression is up-regulated in PMOs cocultured with MDA PCa 2b cells. It is noteworthy that up-regulation of *Cbfa1* transcripts was not always observed when *Osteocalcin* was induced in cocultured PMOs. After PMOs and prostate cancer cells had been separated, a 2-fold increase in alkaline phosphatase activity was seen in PMOs that had been grown with MDA PCa 2a or MDA PCa 2b cells. In contrast, decreased alkaline phosphatase activity was detected when PMOs had been cocultured with PC3 cells, compared with PMOs grown alone (Fig. 4). Von Kossa staining (Fig. 5) shows an increase in calcified matrix forma-

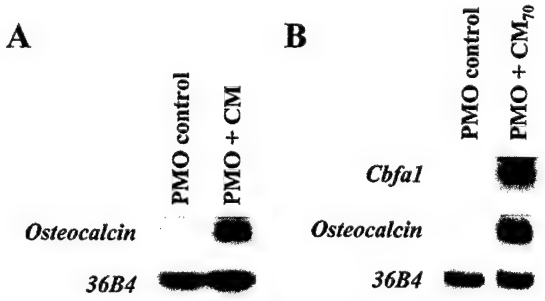



Fig. 6. A. Northern blot analysis of *Osteocalcin* expression in PMOs grown for 6 h in growth medium alone (*control*) or with CM obtained from MDA PCa 2b cells; these experiments were performed in serum-free conditions. *B,* Northern blot analysis of *Cbfa1* and *Osteocalcin* expression in PMOs grown for 12 h in growth medium alone (*control*) or in growth medium supplemented with $CM_{70}$. *36B4* was used as a loading control.

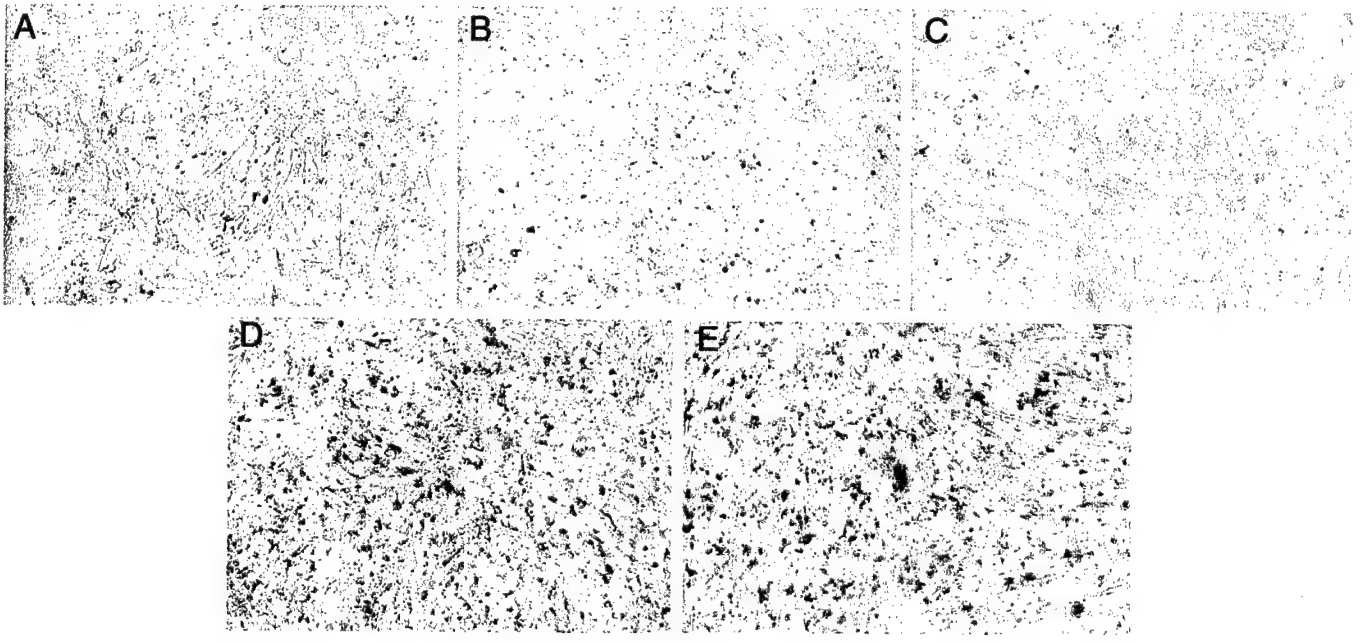



Fig. 5. To determine calcified matrix deposition, von Kossa staining was applied to PMOs after they had been grown in differentiation medium containing BMP-2 for 16 days. *A,* PMOs grown alone; *B,* PMOs grown with LNCaP cells; *C,* PMOs grown with PC3 cells; *D,* PMOs grown with MDA PCa 2b cells; *E,* PMOs grown with MDA PCa 2a cells.

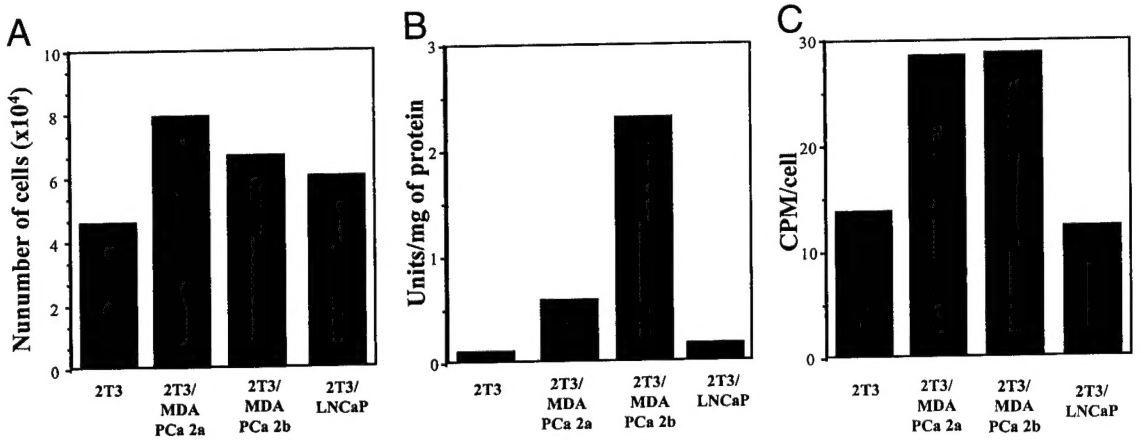



Fig. 7. Proliferation and markers of differentiation of the mouse osteoblast cells 2T3 grown alone (*2T3*) and after 4 days in coculture with MDA PCa 2a, MDA PCa 2b, and LNCaP cells. The cells were harvested and counted with a hematocytometer. The controls and each different coculture were assayed in triplicates. Alkaline phosphatase activity (units/mg protein) was measured with a Sigma Chemical Co. Diagnostics kit. Osteocalcin was evaluated by performing an immunoradiometric assay (Immunotopics, Inc).

tion in PMOs that had been cocultured with MDA PCa 2a or with MDA PCa 2b, compared with PMOs grown alone. PMOs cocultured with LNCaP cells did not induce calcified matrix formation, and PMOs cocultured with PC3 cells exhibited a decrease in calcified matrix formation, compared with controls. This suggests that PC3 cells secrete molecules that inhibit the osteoblast differentiation program. These results agree with those of others (25, 26) and further validate this system.

**Soluble Factors Produced by MDA PCa 2b Cells Induced Cbfa1 and Osteocalcin Expression in PMOs.** MDA PCa 2b cells were able to induce expression of markers of osteoblast differentiation by PMOs in the coculture system. Because in this coculture system, prostate cancer cells and PMOs are not in physical contact, it is likely that soluble factor (or factors) secreted by these prostate cancer cells mediates this effect. We therefore assessed the effect of CM produced by MDA PCa 2b cells on the expression of *Cbfa1* and *Osteocalcin* by PMOs. Fig. 6*A* shows a substantial increase in *Osteocalcin* expression after 6 h of PMOs grown in crude CM. When PMOs were grown for 12 h in α-MEM plus 3 × $CM_{70}$, a substantial increase in *Cbfa1* and *Osteocalcin* expression was observed (Fig. 6*B*). These results indicate that soluble factors produced by MDA PCa 2b cells induce expression of *Cbfa1* and in turn *Osteocalcin*, a target of *Cbfa1*.

**Coculturing with Prostate Cancer Cell Lines Induces Proliferation and Differentiation of Immortalized Murine Osteoblasts (2T3 Cells).** Results are summarized in Fig. 7. Increased 2T3 proliferation, alkaline phosphatase activity, and osteocalcin production were detected when these cells were cocultured with MDA PCa 2a and MDA PCa 2b cells, compared with controls. A significant increase in 2T3 growth, but no increased alkaline phosphatase activity or osteocalcin production was detected when 2T3 cells were cocultured with LNCaP cells.

**Gene Expression Analysis.** To study the molecular changes underlying the stimulation effect on osteoblast growth and differentiation induced by MDA PCa 2a and MDA PCa 2b cells, gene array analysis was performed. This analysis revealed that >7500 genes and expressed sequence tags showed measurable hybridization signals with both PMO controls and PMOs cocultured with MDA PCa 2b cells. The expression of 30 genes was increased ≥5-fold, and the expression of 44 genes was decreased ≥5-fold in PMOs after coculturing.[6] Among those genes, two families were of interest: extracellular matrix genes, including all procollagens tested, showed increase expression (Table 1), whereas transcripts associated with myoblast differentiation showed decreased expression (Table 2). Osteoblasts are bone-forming cells that, once terminally differentiated, produce most of the extracellular bone matrix, which is composed of collagenous and noncollagenous proteins. The main collagen produced by osteoblasts is procollagen type I, which accounts for 90% of the protein content of the bone matrix (27). Among the noncollagenous matrix proteins, bone sialoprotein and osteopontin are the most abundant. We performed a Northern blot analysis using RNA from another coculture experiment to confirm this modulation of gene expression. Fig. 8*A* illustrates that *Procollagen type I* was up-regulated in PMOs cocultured with MDA PCa 2b cells. In contrast, no up-regulation of *Procollagen type I* was observed when PMOs were grown in the presence of LNCaP or PC3 cells. Fig. 8*B* shows that PMOs grown in the presence of MDA PCa 2b cells up-regulated *Osteopontin* expression, confirming the results from the gene array study. Expression of *Myosin light chain 2* and *Myoglobin* was decreased in PMOs cocultured with MDA PCa 2b cells, compared with PMOs grown alone or in the presence of PC3 cells (Fig. 8, *C* and *D*). Taken together, these results indicate that MDA PCA 2b cells favor molecular events that led to osteoblast differentiation, while inhibiting myoblast differentiation.

**MDA PCa 2b Cells Produced Osteoblastic Lesions *in Vivo*.** All mice that had undergone intrabone injections with MDA PCa 2b cells had increased PSA levels. The median PSA blood levels were 4.5 ng/ml and 12 ng/ml at days 21 and 45, respectively, after the injections. None of the control mice tested had blood PSA levels >0.2 ng/ml Bone lesions were monitored by X-ray at days 60, 80, and 100. Three mice died before X-ray was performed. Of the remaining 10 mice, 5, 6, and 8 developed bone (mostly osteoblastic) lesions at days 60, 80, and 100, respectively, after the injection. The contralateral legs never showed evidence of osteoblastic lesions. The mice also exhib-

Table 1 *Gene expression of extracellular matrix genes*

| Balanced differential expression | Gene name[a] |
|---|---|
| 6.0 | *Mus musculus* mRNA for *collagen a1(V)* |
| 9.5 | *Procollagen, type XI, α 1* |
| 9.5 | *Procollagen, type III, α 1* |
| 11.2 | *Procollagen, type VI, α 1* |
| 12.3 | *Secreted phosphoprotein 1 (Osteopontin)* |
| 18.9 | *Procollagen, type V, α 2* |
| 22.5 | *Procollagen, type I, α 1* |

[a] Reviewed in Ref. (27).

[6] Internet address: www.mdanderson.org/PMOGeneExpression.

Table 2 *Expression of genes related to myoblast differentiation*

| Balanced differential expression | Gene name | Reference |
|---|---|---|
| −24.8 | *Mus musculus myosin light chain 2* | Xu *et al.*, 2000 (47) |
| −10.8 | *Muscle glycogen phosphorylase* | Froman *et al.*, 1998 (48) |
| −11.9 | *Mus musculus* mRNA for stretch-regulated skeletal muscle protein (*Usmg4* gene) | Xu *et al.*, 2000 (47) |
| −10.7 | *Myoglobin* | Garry *et al.*, 2000 (49) |
| −6.2 | *Enolase 3, β* muscle | Keller *et al.*, 1992 (50) |
| −6.9 | *Ryanodine* receptor 1, skeletal muscle | Ogawa *et al.*, 1999 (51) |

ited features of bone resorption, primarily at the injection site (Fig. 9). One mouse had a rapidly growing soft tissue tumor and died soon after the first X-ray, and only 1 had no evidence of tumor. Nineteen control mice had $1 \times 10^6$ PC3 prostate cancer cells injected under the same conditions. X-ray was performed in every mouse 30–60 days after the procedure. None of the 14 mice that survived the procedure for at least 1 month developed osteoblastic lesions, and 12 (86%) rapidly developed osteolytic lesions (Fig. 9). Histopathological examination showed evidence of new bone formation when the bones had been injected with MDA PCa 2b. The left side of Fig. 10*B* shows MDA PCa 2b cells growing in the femoral shaft of the injected limb, surrounded by woven (immature) bone, which is typical of rapid bone formation. Surrounding the new bone is the lamellar (mature) bone. In the right side of Fig. 10*B*, higher magnification reveals the randomly deposited basket-weave pattern that is characteristic of new bone formation or bone remodeling (immature bone). In contrast with what occurred with MDA PCa 2b cells, osteoclast recruitment and bone destruction were obvious in bones that had been injected with PC3 cells (Fig. 10*C*). The left side of Fig. 10*C* shows a tumor produced by PC3 cells growing in the marrow cavity of a severe combined immunodeficiency disease mouse. The tumor has destroyed cortical bone and is growing into the muscle outside the bone. In the right side of the same panel, higher magnification allows identification of several osteoclasts, a characteristic finding in bone injected with PC3 cells.

## DISCUSSION

Currently, no model is available to study the cellular and molecular events associated with bone metastases of prostate cancer. This study shows that MDA PCa 2a and MDA PCa 2b cells induce a specific and reproducible increase in osteoblast differentiation and proliferation when the cells share the medium during coculturing. Osteoblast differentiation in this system was associated with up-regulation of the osteoblast-specific transcriptor factor *Cbfa1*. Moreover, up-regulation of *Cbfa1* and *Osteocalcin* expression was also induced in PMOs by CM produced by MDA PCa 2b cells, suggesting that soluble factors produced by prostate cancer cells promote osteoblast differentiation and that *Cbfa1* mediates this effect. To our knowledge, this is the first *in vitro* model of bone metastasis from prostate cancer that recapitulates the osteoblastic phenotype typical of the disease. These results confirmed *in vivo* and at the molecular level, suggest that the pathophysiology of osteoblastic bone metastases from prostate cancer is related to an increase in bone formation rather than (or in addition to) a decrease in bone destruction.

The development, differentiation, and maturation of the osteoblast phenotype in primary cultures of rat calvaria osteoblasts are well characterized (20–22). Recent reports have also documented expression of markers of osteoblast differentiation in PMOs (23, 24). The primary cultures used in this study showed high levels of *Cbfa1* transcriptional activity and *Osteocalcin* expression, which indicate that PMOs are composed of osteoblasts and their precursors. MDA PCa 2a and MDA PCa 2b cells up-regulate markers of osteoblast differentiation, namely expression and secretion of *Osteocalcin,* secretion of alkaline phosphatase, and formation of bone matrix. We also detected increased expression of *Cbfa1* in PMOs cocultured with MDA PCa 2b cells, which suggests that *Cbfa1* mediates osteoblast differentiation in this system. Increased expression of *Cbfa1* transcripts was not always observed in cocultured PMOs when *Osteocalcin* was up-regulated. Recently, it has been proposed that *Cbfa1* transcriptional activity be regulated directly by transcription factors, that *Cbfa1* may need to be activated by posttranscriptional modifications, and that *Cbfa1* function could be modulated by cofactors (28); therefore, it is likely that *Cbfa1* activation occurs in the absence of up-regulation of its transcripts. Gene expression analysis of PMOs grown in the presence of MDA PCa 2b cells showed that soluble factors produced by these prostate cancer cells induced a shift in the expression of transcripts, resulting in increased expression of extracellular matrix genes and decreased expression of genes related to myoblast differentiation. Osteoblasts are derived from common mesenchymal progenitors that can also differentiate into chondroblasts, myoblasts, and adipocytes (29–31). Moreover, some clones derived from rat calvaria osteoblasts have been reported to exhibit both osteoblast-like phenotypes and notable formation of myotubes (30, 31). This indicates that osteoblasts derived from neonatal rodent calvaria are in different stages of differentiation and developmental commitment, which probably accounts for the expression of *Myosin light chain 2* and *Myoglobin* in PMOs. The possibility of contamination by myoblasts from surrounding muscle in PMO cultures is unlikely because the procedure was designed and validated to remove nonbone cells (13). The decreased levels of transcripts of genes related to myoblast differentiation in PMOs after coculturing with MDA PCa 2b cells are more likely the result of the enrichment of the PMOs either by increased proliferation of cells of the osteoblast lineage or by selective inhibition of myoblast differentiation. Myogenic differentiation has been reported to be inhibited by factors such as BMP-2, transforming growth factor-$\beta$, and basic-fibroblast growth factors (32–34). Moreover, BMP-2 has been shown to convert differentiation of myoblasts into the osteoblast lineage (35). Human prostate cancer cells have been shown to produce several growth regula-

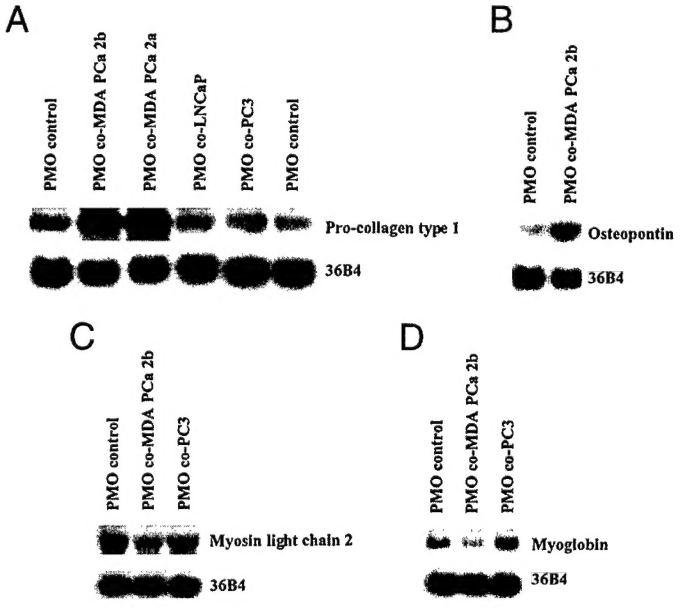



Fig. 8. Northern blot analysis of expressed genes in PMOs grown alone (*PMO control*) or after 4 days of coculturing with MDA PCa 2a, MDA PCa 2b, LNCaP, or PC3 cell lines. *A, Procollagen type I* expression; *B, Osteopontin* expression; *C, Myosin light chain 2* expression; *D, Myoglobin* expression. *36B4* was used as a loading control.

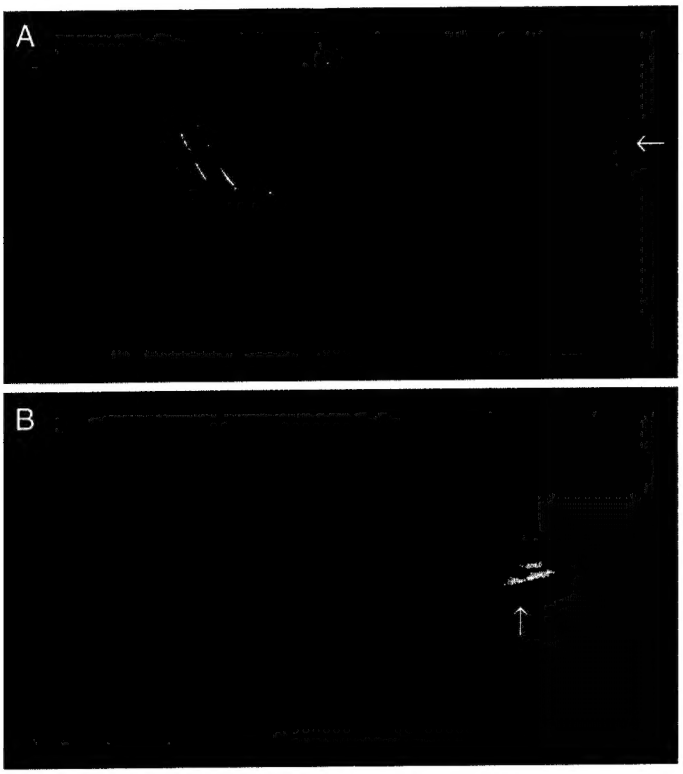



Fig. 9. X-ray imaging of mouse legs 6 weeks after intrabone injection of prostate cancer cells. *A*, thinning of cortical bone after injection of PC3 cell, suggesting increased bone resorption. *B*, increased bone density after injection of MDA PCa 2b cell.

tory factors, including transforming growth factor-$\beta$ and basic-fibroblast growth factors, as well as bone morphogenetic proteins (35), again suggesting that BMP-2 may be a player in the osteoinductive effect of prostate cancer cells.

Our findings of an osteoinductive effect of MDA PCa 2a and MDA PCa 2b cells *in vitro* were reproducible in an *in vivo* model. Osteoclasts were scarce in tumors produced by MDA PCa 2b cells, whereas tumors produced by PC3 cells resulted in osteolytic lesions and substantial recruitment of osteoclasts. Until recently, our insight into bone-metastasis biology was based mostly on the study of prostate cancer cell lines. These studies were limited because: (*a*) the bone compartment was not studied; and (*b*) the cell lines used (mainly LNCaP, PC3, and DU-145) do not fully reflect the common biological features of bone metastasis from prostate cancer (8). Geldof and Rao (36) have reported that injection of R3327-MatLyLu rat tumor cells into the tail vein, with concomitant vena cava occlusion, results in skeletal metastases, although these lesions are osteolytic. Finally, Wu *et al.* (37) have reported that two of seven animals developed osteoblastic bone metastases after receiving intracardiac injection of C4–2 cells derived from LNCaP. However, because the growth of C4–2 cells is not regulated by androgen, we believe our system provides the *in vitro* model counterpart to these models and will be useful in identifying bone metastasis-related genes, osteoblast-stimulating factors, or both, which might be more relevant to the natural history of metastatic prostate cancer in humans.

Our work confirms that modulation of osteoblast proliferation by prostate cancer cells occurs without any physical contact. Gutman *et al.* (38) first hypothesized the production of osteoblast-stimulating factors by prostate cancer, and our results agree thus far. Indeed, PSA (39, 40), urokinase (41, 42), bone morphogenic proteins (43, 44), and endothelin-1 (45) have been identified as direct or indirect osteoblast-stimulating factors expressed by prostate cancer cells. Of note, the role of some of these factors (41, 46) has been established by use of the conditioned culture medium of PC3, a cell line that does not reflect the common biological features of prostate cancer, because it is minimally differentiated and does not produce PSA. Our work confirms that PC3, in contrast to MDA PCa 2b, does not produce osteoblastic lesions *in vivo* but actually produces osteolytic lesions.

In contrast to the consistent and reproducible induction of osteoblastic growth observed in our *in vitro* model, we often saw increased proliferation of MDA PCa 2a and MDA PCa 2b cells, but it did not always reach statistical significance. These results might indicate that other cells from the bone compartment are required to provide a growth advantage to prostate cancer cells. The conditions under which we performed these experiments were set to optimize the study of PMO growth and differentiation, but they might not have been optimal for studying the proliferative response of prostate cancer cells in the presence of PMOs. Finally, the short duration of the coculturing in our system may be an alternative explanation for the apparent lack of a reproducible increase in prostate cancer cell growth, in terms of their well-recognized slow growth.

In summary, our results describe the establishment, optimization, and molecular analysis of an *in vitro* model of bone metastases from prostate cancer. MDA PCa 2a and MDA PCa 2b cells induced the osteoblastic features of bone metastases observed in the clinic, and this effect was mediated by increased osteoblastic growth and differentiation. This model proved valuable for studying molecular mechanisms underlying the interactions of prostate cancer and bone. It may also represent an attractive system for identifying molecular targets, on either the malignant compartment or the osteoblast compartment that may prevent the growth of prostate cancer cells in bone.

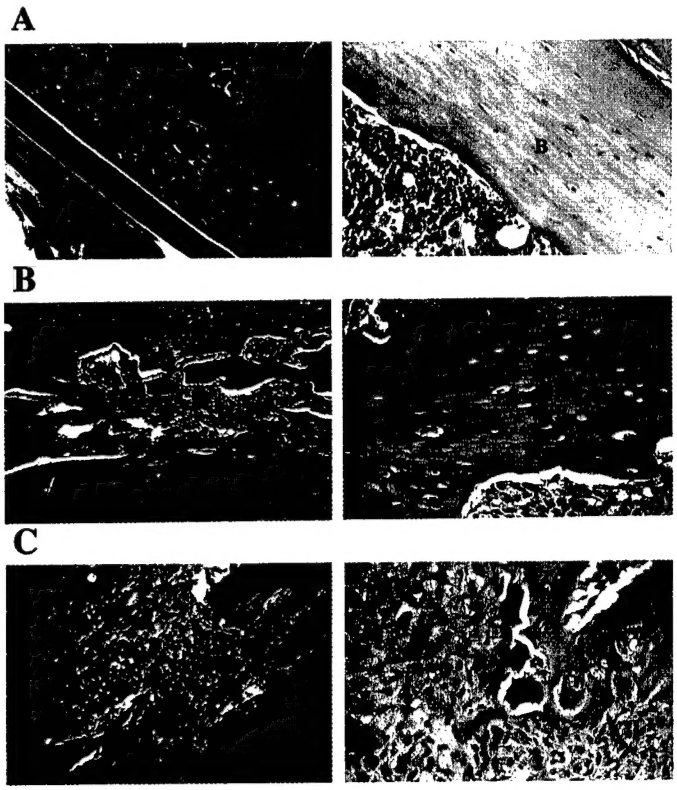



Fig. 10. *A*, longitudinal section of mouse femur stained with H&E (*left*: ×40, *right*: ×200). *Right panel*, normal bone with lamellar pattern, which is seen after normal bone formation (*i.e.*, old or mature bone). *B*, mouse femur after intrabone injection of MDA PCa 2b cells showing microscopic evidence of new bone formation. *C*, histological evidence of osteolytic lesions in the femur of a mouse injected with PC3 cells. Osteoclasts (*arrows*) are obvious at the tumor bone interface. *M*, bone marrow; *B*, cortical bone; *T*, tumor; *WB*, woven (immature) bone; *LB*, lamellar (mature) bone.

## REFERENCES


1. Greenlee, R. T., Hill-Harmon,. M. B., Murray, T., and Thun, M. Cancer statistics. CA Cancer J. Clin., *51:* 15–36, 2001.
2. Scardino, P. T. Early detection of prostate cancer. Urol. Clin. North Am.. *16:* 635–655, 1989.
3. Catalona, W. J. Management of cancer of the prostate. N. Engl. J. Med., *331:* 996–1004, 1994.
4. Cook, G. B., and Watson, F. R. Events in the natural history of prostate cancer: using salvage curves, mean age distributions and contingency coefficients. J. Urol.. *96:* 87–96, 1968.
5. Paget, S. The distribution of secondary growth in cancer of the breast. Lancet, *1:* 571–573, 1989.
6. Hart, I. R. 'Seed and soil' revised: mechanisms of site-specific metastasis. Cancer Metastasis Rev., *1:* 5–16, 1982.
7. Koeneman, K. S., Yeung, F., and Chung, L. W. K. Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. Prostate, *39:* 246–261, 1999.
8. Navone, N. M., Logothetis, C. J., von Eschenbach A. C., and Troncoso, P. Model systems of prostate cancer: uses and limitations. Cancer Metastasis Rev., *17:* 361–371, 1999.
9. Royai, R., Lange, P. H., and Vessella, R. Preclinical models of prostate cancer. Semin. Oncol., *23:* 35–40, 1996.
10. Navone, N. M., Olive, M., Ozen, M., Davis, R., Troncoso, P., Tu, S-M., Johnson, D., Pollack, A., Pathak, S., von Eschenbach, A. C., and Logothetis, C. J. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. Clin. Cancer Res., *3:* 2493–2500, 1997.
11. Navone, N. M., Rodriguez-Vargas, M. del C., Benedict, W. F., Troncoso, P., McDonnell, T. J., Zhou, J-H., Luthra, R., and Logothetis, C. J. TabBO: a model reflecting common molecular features of androgen-independent prostate cancer. Clin. Cancer Res., *6:* 1190–1197, 2000.
12. Ghosh-Choudhury, N., Windle, J. J., Koop, B. A., Harris, M. A., Guerrero, D. L., Wozney, J. M., Mundy, G. R., and Harris, S. E. Immortilazed murine osteoblast derived from BMP 2-T-antigen expressing transgenic mice. Endocrinology, *337:* 331–339, 1996.
13. Zhang, R., Ducy, P., and Karsenty, G. 1,25-dihydroxyvitamin D3 inhibits osteocalcin expression in mouse through an indirect mechanism. J. Biol. Chem., *272:* 110–116, 1997.
14. Ducy, P., and Karsenty, G. Two distinct osteoblast-specific *cis*-acting elements control expression of a mouse osteocalcin gene. Mol. Cell. Biol., *15:* 1858–1869, 1995.
15. Bharagava, U., Bar-Lev, M., Bellows, C. G., and Aubin, J. E. Ultrastructural analysis of bone nodules formed *in vitro* by isolated fetal rat calvaria cells. Bone, *9:* 155–163, 1986.
16. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell, *89:* 747–754, 1997.
17. Laborda, J. 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. Nucleic Acids Res., *19:* 3998, 1991.
18. Karsenty, G. The genetic transformation of bone biology. Genes Dev., *13:* 3037–3051, 1999.
19. Navone, N. M., Yang, J., Olive, M., Wu, X., Palmer, J. L., Logothetis, C. J., and Karsenty, G. Expression of an osteoblast-specific transcription factor following co-culture with prostate cancer bone metastases. Proc. Am. Assoc. Cancer Res., *40:* 328, 1999.
20. Bellows, C. G., Aubin, J. E., Heersche, J. N. M., and Antosz, M. E. Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. Calcif. Tissue Int., *38:* 143–154, 1986.
21. Bellows, C. G., and Aubin, J. E. Determination of numbers of osteoprogenitors present in isolated fetal rat calvaria cells *in vitro*. Dev. Biol., *133:* 8–13, 1989.
22. Malaval, L., Liu, F., Roche, P., and Aubin, J. E. Kinetics of osteoprogenitor proliferation and osteoblast differentiation *in vitro*. J. Cell. Biochem., *74:* 616–627, 1999.
23. Thomas, G. P., Bourne, A., Eisman, J. A., and Gardiner, E. M. Species-divergent regulation of human and mouse osteocalcin genes by calciotropic hormones. Exp. Cell Res., *258:* 395–402, 2000.
24. Mansukhani, A., Bellosta, P., Sahni, M., and Basilico, C. Signaling by fibroblast growth factor (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induced apoptosis in osteoblasts. J. Cell Biol., *149:* 1297–1308, 2000.
25. Martinez, J., Silva, S., and Santibanez, J. F. Prostate-derived soluble factors block osteoblast differentiation in culture. J. Cell. Biochem., *61:* 18–25, 1996.
26. Kido, J-I., Yamauchi, N., Ohishi, K., Kataoka, M., Nishikawa, S., Nakamura, T., Kadono, H., Ikedo, D., Ueno, A., Nonomura, N., Okuyama, A., and Nagata, T. Inhibition of osteoblast cell differentiation by conditioned medium derived from the human prostatic cancer cell line PC-3 *in vitro*. J. Cell. Biochem., *67:* 248–256, 1997.
27. Karsenty, G. Genetics of skeletogenesis. Dev. Genet., *22:* 301–313, 1998.
28. Ducy, P. CBFA1: a molecular switch in osteoblast biology. Dev. Dyn., *219:* 461–471, 2000.
29. Grigoriadis, A. E., Heersche, J. N., and Aubi, J. E. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J. Cell. Biol., *106:* 2139–2151, 1988.
30. Yamaguchi, A., and Kahn, A. J. Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif. Tissue Int., *49:* 221–225, 1991.
31. Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J. M., Rosen, V., Wang, E. A., Kahn, A. J., Suda, T., and Yoshiki, S. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation *in vitro*. J. Cell Biol., *113:* 681–687, 1991.
32. Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J. Cell. Biol., *127:* 1755–1766, 1994.
33. Lee, M. H., Javed, A., Kim, H. J., Shin, H. I., Gutierrez, S., Choi, J. Y., Rosen, V., Stein, J. L., van Wijnen, A. J., Stein, G. S., Lian, J. B., and Ryoo, H. M. Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor beta1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. J. Cell. Biochem., *73:* 114–125, 1999.
34. Shin, C. S., Lecanda, F., Sheikh, S., Weitzmann, L., Cheng, S. L., and Civitelli, R. Relative abundance of different cadherins defines differentiation of mesenchymal precursors into osteogenic, myogenic, or adipogenic pathways. J. Cell. Biochem., *78:* 566–577, 2000.
35. Yamaguchi, A., Komori, T, and Suda, T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr. Rev., *21:* 393–411, 2000.
36. Geldof, A. A., and Rao, B. R. Prostatic tumor (R3327) skeletal metastasis. Prostate, *16:* 279–290, 1990.
37. Wu, T. T., Sikes, R. A., Cui, Q., Thalmann, G. N., Kao, C., Murphy, C. F., Yang, H., Zhau, H. E., Balian, G., and Chung, L. W. Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. Int. J. Cancer, *77:* 887–894, 1998.
38. Gutman, E. B., Sproul, E. E., and Gutman, A. B. Significance of increased phosphatase activity of bone at the site of osteoplastic metastases secondary to carcinoma of the prostate gland. Am. J. Cancer, *28:* 485–495, 1936.
39. Killian, C. S., Corral, D. A., Kawinski, E., and Constantine, R. I. Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent TGF$\beta$ and a proteolytic modulation of cell adhesion receptors. Biochem. Biophys. Res. Commun., *192:* 940–947, 1993.
40. Cramer, S. D., Chen, Z., and Peehl, D. M. Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTH-rP stimulated cAMP accumulation in mouse osteoblasts. J. Urol., *156:* 526–531, 1996.
41. Rabbani, S. A., Desjardins, J., Bell, A. W., Banville, D., Mazar, A., Goltzman, D. An amino-terminal fragment of urokinase isolated from a prostate cancer cell line (PC-3) is mitogenic for osteoblast-like cells. Biochem. Biophys. Res. Commun., *173:* 1058–1064, 1990.
42. Achbarou, A., Kaiser, S., Tremblay, G., Ste-Marie, L. G., Brodt, P., Goltzman, D., and Rabbani, S. A. Urokinase overproduction results in increased skeletal metastasis by prostate cancer cells *in vivo*. Cancer Res., *54:* 2372–2377, 1994.
43. Harris, S. E., Harris, M. A., Mahy, P., Wozney, J., Feng, J. Q., and Mundy, G. R. Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells. Prostate, *24:* 204–211, 1994.
44. Bentley, H., Hamdy, F. C., Hart, K. A., Seid, J. M., Williams, J. L., Johnstone, D., and Russell, R. G. G. Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia. Br. J. Cancer, *66:* 1159–1163, 1992.
45. Nelson, J. B., Hedican, S. P., George, D. J., Reddi, A. H., Piantadosi, S., Eisenberger, M. A., and Simons, J. W. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nat. Med., *1:* 944–949, 1995.
46. Perkel, V. S., Subburaman, M., Herring, S. J., Baylink, D. J., and Linkhart, T. A. Human prostatic cancer cells, PC-3, elaborate mitogenic activity which selectively stimulates human bone cells. Cancer Res., *50:* 6902–6907, 1990.
47. Xu, Y., He, J., Wang, X., Lim, T. M., and Gong, Z. Asynchronous activation of 10 muscle-specific protein (MSP) genes during zebrafish somitogenesis. Dev. Dyn., *219:* 201–215, 2000.
48. Froman, B. E., Tait, R. C., and Gorin, F. A. Role of E and CArG boxes in developmental regulation of muscle glycogen phosphorylase promoter during myogenesis. DNA Cell Biol., *17:* 105–115, 1998.
49. Garry, D. J., Meeson, A., Yan, Z., and Williams, R. S. Life without myoglobin. Cell Mol. Life Sci., *57:* 896–898, 2000.
50. Keller, A., Ott, M. O., Lamande, N., Lucas, M., Gros, F., Buckingham, M., and Lazar, M. Activation of the gene encoding the glycolytic enzyme beta-enolase during early myogenesis precedes an increased expression during fetal muscle development. Mech. Dev., *38:* 41–54, 1992.
51. Ogawa, Y., Kurebayashi, N., and Murayama, T. Ryanodine receptor isoforms in excitation-contraction coupling. Adv. Biophys., *36:* 27–64, 1999.